



PHD

Mechanisms involved in ulceration of the stomach and small bowel

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Award date:
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**MECHANISMS INVOLVED IN ULCERATION OF THE STOMACH AND
SMALL BOWEL**

submitted by Julia Reid B.Sc.
for the degree of PhD
of the University of Bath
1998

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DEDICATION

To My Father

John Henry Rudman

30.11.13 - 8.03.67

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ACKNOWLEDGEMENTS

I am grateful to my supervisors, Dr. Lidia Notarianni and Dr. Anthony Collins for providing me with the opportunity to work towards a Ph.D., also for their help and guidance, and to SmithKline Beecham for funding this research.

I would also like to thank my friends and colleagues from the Area Central Laboratories (ACL) and the Wolfson Research Centre (WRC) at the Royal United Hospital in Bath, and at the University of Bath, without whose advice, assistance and generosity much of this work would not have been possible. In particular I would like to thank:

Ralph Nunn FIMBS, Carolynn Male and Jackie Price (Histopathology, ACL)

Keith Moss and Yvonne Cartwright (Biochemistry, ACL)

Andrew Smith and Alan Tongue (Haematology, ACL)

Clive Haynes (Immunology, ACL)

Diane Belk (Clinical Pharmacology, WRC)

Chris Stirling (Diabetes and Lipid Research, WRC)

Philip Bell, Alison Burlton and Dr. Andrew Bosenquet (Bath Cancer Research, WRC)

Ursula Potter (The Centre for Electron Optical Studies, University of Bath)

Finally thanks to Ken, my husband, for his unstinting help and support.

ABBREVIATIONS

ATP	adenosine triphosphate
ADP	adenosine diphosphate
BC	Bath Clinic
BPAA	[1,1'-biphenyl]-4-acetic acid
CINC	cytokine-induced neutrophil chemoattractant
COX	cyclooxygenase
DAB	3,3'-diaminobenzidine
EGF	epidermal growth factor
ELAM	endothelial leucocyte adhesion molecule
FFPE	formalin-fixed paraffin-embedded
FMLP	N-formylmethionylleucylphenylalanine
GM-CSF	granulocyte-macrophage-colony stimulating factor
IFN	interferon
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
ICAM	intercellular adhesion molecule
LFA	leucocyte functional antigen
LT	leukotriene
MAC	macrophage antigen
M-CSF	macrophage-colony stimulating factor
6MNA	6-methoxy-2-naphthylacetic acid
NSAID	nonsteroidal antiinflammatory drug
PG	prostaglandin
RNHRD	Royal National Hospital for Rheumatic Diseases
ROMS	reactive oxygen metabolites
RUH	Royal United Hospital
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TNF	tumour necrosis factor
VIP	vasoactive intestinal polypeptide

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SUMMARY

An investigation into the mechanisms involved in ulceration of the stomach and small bowel was conducted. Wistar rats were dosed with a selection of NSAIDs, diclofenac, fenbufen, indomethacin, nabumetone, naproxen and piroxicam, at varying concentrations over different periods of time (one to twenty-eight days) and the corresponding effects on the stomach and small bowel were investigated. NSAID-induced ulceration of the rat small bowel, caused only by indomethacin or diclofenac, was found to be associated with bacteria.

The bacteria associated with rat small bowel ulcers were investigated via scanning electron microscopy. Attempts at identification were conducted via the use of the antibiotics, amoxycillin, cloxacillin, erythromycin, metronidazole, nalidixic acid and neomycin sulphate, *in vivo*, and via antibiotic susceptibility tests *in vitro*. Numerous bacteria were isolated but the only ones positively identified were *Proteus spp.* and *Escherichia coli*.

The effects of indomethacin on a cellular level, within the small bowel mucosa of rats, were examined via immunohistochemistry in order to try and determine the role of cytokines in ulcer formation. The concentration of TNF- α was found to be highest within the Peyer's patches associated with ulcers and lowest within the un ulcerated regions, between ulcers, in small bowel tissue taken from rats dosed with indomethacin ID₆₀. The activity of TNF- α within normal control rat small bowel tissue was approximately midway between the two extremes observed within the ulcerated tissue. TNF- α activity in the small bowel tissue of animals treated with indomethacin ID₃₀, a non-ulcerogenic dose, was approximately half that observed within the control tissue. Concentrations of IL-1 α were found to be highest within the small bowel tissue taken from rats dosed with indomethacin ID₃₀, and lowest within the ulcerated small bowel tissue. IL-1 α activity within the normal control tissue was approximately half that observed within indomethacin ID₃₀ tissue and twice that observed in the ulcerated small bowel tissue.

The concentrations of serum haptoglobin and serum myoglobin within the systemic circulation of rats treated with the selected NSAIDs were investigated in order to ascertain their usefulness as *in vivo* protein markers of the presence and severity of small bowel ulceration.

Helicobacter pylori-positive and *Helicobacter pylori*-negative human gastric mucosa samples, from patients attending The Royal National Hospital of Rheumatic Diseases in Bath, were examined via immunohistochemistry. The activity of the of cytokines TNF- α , IL-1 α and IL-8 were found to be elevated within the infected gastric mucosa tissue.

1.0 INTRODUCTION

1.1 PHYSIOLOGY AND PATHOLOGY OF THE STOMACH AND SMALL BOWEL

1.1.1 Stomach

The stomach has a number of characteristics which enable it to perform various functions (see fig. 1.1.1). The main receptacle of the stomach consists of the fundus and the body which are between them able to expand and accommodate an increase in volume of up to 1.5 litres without an equivalent increase in intragastric pressure. The contractions of the fundus and body are usually weak so most of the gastric contents here remain relatively unmixed for long periods. In the antrum, however, the contractions are vigorous, fulfilling the dual role of thoroughly mixing the antral chyme with gastric juice and emptying, in small bursts, the contents of the stomach into the duodenal bulb.

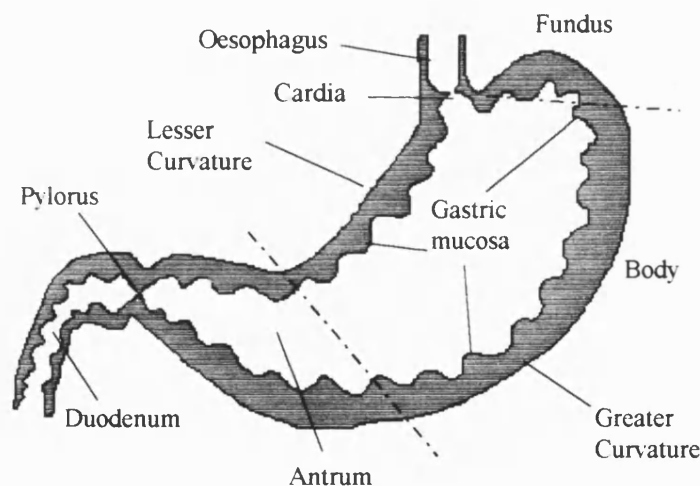


Fig. 1.1.1 The Major Anatomical Divisions of the Stomach

The gastric mucosa is comprised of many deep glands which in the body and the fundus contain parietal (or oxyntic) cells, which secrete hydrochloric acid and intrinsic factor, and chief (or zymogen) cells which secrete pepsinogens. Several of these glands open onto a common chamber called the gastric pit. In the antral region of the stomach the glands contain G cells which release gastrin, a hormone which stimulates gastric acid and pepsin secretion. [Ganong, 1987; Kutchai, 1993a].

1.1.2 The Gastroduodenal Junction

The gastric antrum and the initial part of the duodenum (duodenal bulb or cap) are separated by the pylorus. Although the pylorus is not strictly a true anatomical sphincter it functions physiologically as such in many respects. The circular smooth muscle of the pylorus forms two ring-like thickenings that are followed by a connective tissue ring that separates the pylorus from the duodenum. The pylorus is densely innervated by vagal and sympathetic nerve fibres. The sympathetic postganglionic fibres release norepinephrine which acts on α -adrenergic receptors causing the sphincter to constrict even more. The vagal fibres have both excitatory and inhibitory effects on the smooth muscle of the pylorus. Cholinergic vagal fibres stimulate the sphincter to constrict and the inhibitory vagal fibres release another transmitter, vasoactive intestinal polypeptide (VIP), which causes it to relax. In addition to this other substances, cholecystokinin, gastrin, gastric inhibitory peptide and secretin cause the pylorus to constrict. It is not known if all these hormones are physiological regulators for the pylorus, however, cholecystokinin constricts the pyloric sphincter at concentrations which activate gall-bladder constriction.

This junction has two functions, it regulates the emptying of the stomach, so that the gastric contents pass through to the duodenum at a rate that the duodenum can process the chyme, as too rapid emptying can lead to duodenal ulcers. It also prevents regurgitation of the duodenal contents back into the stomach as although the gastric mucosa is highly resistant to acid it may be damaged by bile. [Ganong, 1987; Kutchai, 1993b].

1.1.3 The Small Bowel

The small bowel, particularly the duodenum and jejunum, is the site of most digestion and absorption. It is approximately five metres long, is the largest segment of the gastrointestinal tract and accounts for 75 per cent of the total length in humans (the chyme takes between two to four hours to traverse the entire length). About nine litres of fluid pass through the small bowel per day, two via fluid intake through the stomach and the rest is made up of gastrointestinal secretions.

1.1.3.1 The Duodenum

The duodenal bulb is the first part of the duodenum and is regularly in contact with the acidic gastric contents which have been emptied from the stomach through the pylorus. As a result this is a common site of peptic ulceration. The duodenum is approximately 25 cm long, is the region without any mesentery, and easily distinguished from the rest of the small bowel histologically. The villi of the distal duodenum may be leaf or finger shaped and range in height from 0.5 to 0.8 mm. [Ganong, 1987; Madara & Trier, 1987a; Kutchai, 1993c].

1.1.3.2 The Jejunum

At the ligament of Treitz the duodenum becomes the jejunum which accounts for approximately 40 per cent of the remainder of the small bowel although there is no sharp dividing line between it and the ileum. The villi in the human proximal jejunum are similar to those found in the distal duodenum. [Ganong, 1987; Madara & Trier, 1987a; Kutchai, 1993c].

1.1.3.3 The Ileum

The ileum is the longest part of the small bowel, accounting for almost 60 per cent of the entire length, and in this region the human villi are finger shaped, rarely exceeding 0.5 mm in height. The distal ileum is the site of reabsorption of bile acids and here the epithelial cells actively take up bile acids against a large concentration gradient. As a result the pH in the distal ileum tends to be higher than the rest of the small bowel [Borgström *et al.*, 1957] which is usually at a pH of 7.0. The terminal ileum is separated from the caecum, the first part of the colon, by the ileocaecal sphincter. [Ganong, 1987; Madara & Trier, 1987a; Kutchai, 1993c].

1.1.4 The General Structure of the Gastrointestinal Tract Wall

The gastrointestinal mucosa is made up of three distinct layers. The deepest is a continuous thin sheet of contractile smooth muscle, ten cells thick, called the muscularis mucosa which separates the mucosa from the submucosa. Its precise role is unclear but it is thought to contribute to intestinal function as its contractions may facilitate the

emptying of crypt luminal contents by causing luminal compression. In addition, it is possible that the contractions also contribute to villi movement thus affecting the unstirred layer adjacent to the absorptive epithelium.

The middle mucosal layer, called the lamina propria, is the continuous connective tissue space which forms the connective tissue core of the villi and surrounds the crypt epithelium. The lamina propria, which supports the epithelial cells, has a number of important functions the main one being that it contains the blood vessels which nourish the epithelium. The most abundant cell types present are the mononuclear cells which are necessary for an immunological response, such as plasma cells, lymphocytes and macrophages, and as a consequence the lamina propria has important immunological functions both in healthy and disease states. The blood and lymph vessels of the lamina propria also transport the material which is absorbed by the epithelium.

The third layer of the intestinal mucosa consists of a continuous sheet of epithelial cells, one cell thick, which lines the villi and the crypts and as a result is in direct contact with the contents of the lumen. The epithelium is separated from the lamina propria by a thin continuous basement membrane which is in two parts, the basal lamina (which is in direct contact with the basal surface of the epithelium) and a deeper layer of reticular fibres and glycoprotein connective tissue (which adheres to the lamina propria). The crypt epithelium consists of many different cell types. Mucus-secreting goblet cells, a variety of endocrine cells, occasionally a caveolated (or tuft) cell, Paneth cells (in most mammals) containing large secretory granules and many undifferentiated cells which are actively proliferating. The epithelium covering the villi is also made up of mucus-secreting goblet cells, rare caveolated cells and a few endocrine cells. However, in addition to these there are large numbers of absorptive cells (or enterocytes). In the ileum there occurs a specialised epithelial cell, the M cell (which covers the top of Peyer's patches) and the occasional cup cell. There are 20 to 40 villi per square mm of mucosa and the free edges of the cells of their epithelium are divided into minute microvilli which form a brush border.

Underlying the mucosa is the submucosa, another layer of connective tissue, which contains a network of nerve cells, called the submucous plexus, as well as blood and lymphatic vessels that penetrate up into the mucosa and down into the muscularis externa. The muscularis externa is made up of two layers of muscle, a relatively thick inner layer of circular muscle and a thinner outer layer of longitudinal muscle, between which lies another network of nerve cells known as the myenteric plexus.

The outermost covering, called the adventitia (in the mesentery free region of the duodenum) or serosa, consists mainly of a thin layer of cells and connective tissue which do not form an integral part of the small bowel. These thin sheets of connective tissue anchor the jejunum and the ileum, via the mesentery, to the posterior body wall, thus supporting the gastrointestinal tract within the abdominal cavity. [Ganong, 1987; Madara & Trier, 1987a; Kutchai, 1993c; Vander *et al.*, 1994; Telford & Bridgman, 1995].

1.2 MECHANISMS OF DAMAGE TO THE STOMACH AND SMALL BOWEL

1.2.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Although NSAIDs have now been in use for almost 100 years some of their modes of action are still not clearly understood. The first evidence that a NSAID could cause damage to the stomach was reported by Douthwaite and Lintott [1938], who observed, via gastroscopy, the damage caused by aspirin, which by this time had been commercially synthesised for at least 40 years [Dreser, 1899]. NSAIDs are now believed to cause this damage in a variety of ways, some of which are interconnected.

1.2.1.1 Ion Trapping

Some of the damage caused to the gastric mucosa by NSAIDs is probably due to a direct, topical effect as they pass through the stomach. Weak organic acids are unable to dissociate in the low pH of the stomach and most NSAIDs are weak acids with pKa values less than 5. Usually such compounds will be 99 per cent ionised in an environment that is two pH units above that of its pKa value but at other times they remain un-ionised. Thus in the stomach, with a pH of 1 to 2, they are un-ionised and in

this form they are able to pass easily through the lipid bilayer that makes up the gastric epithelial cell walls. However, once these weak acids are inside the epithelial cells, where the pH is maintained at between 7.0 and 7.4, they become ionised. The ionised drugs are now trapped inside the cell as they are unable to pass easily back across the phospholipid membrane. This phenomenon of 'ion trapping' [Martin, 1963] means that water soluble NSAIDs can, temporarily, become highly concentrated within the gastric epithelial cells [Brune *et al.*, 1979, 1980] causing damage as a result. The high intracellular concentrations of acidic NSAIDs may cause a direct acid damaging effect [Schoen & Vender, 1989] especially if they are water soluble [Garner, 1978]. Although this high concentration of NSAID is probably short-lived the resulting inhibition of cyclooxygenase (COX) may possibly last for many hours.

1.2.1.2 Re-exposure Due To Enterohepatic Recirculation

The 'ion trapping' mechanism explains some of the damage caused to the gastric mucosa, particularly by water soluble NSAIDs such as aspirin and diclofenac, but does not explain the damage caused to the small bowel which is usually maintained at a neutral pH. The topical damage here is due, in part, to enterohepatic circulation as some NSAIDs and/or their metabolites, once they have been conjugated in the liver, are excreted in the bile. Hydrolysis of these biliary excreted conjugates by bacterial β -glucuronidase and sulphatase result in the small bowel being repeatedly exposed to the active drug enabling some of it to be reabsorbed [Kent *et al.*, 1969; Rainsford, 1983; Simon & Gorbach 1986, 1987; Rainsford, 1989; Bjarnason & MacPherson, 1989]. Evidence supporting this proposed mechanism comes from animal experiments whereby damage to the gastrointestinal mucosa caused by indomethacin can either be greatly reduced upon fasting, which decreases bile flow; or prevented by bile duct ligation [Brodie *et al.*, 1970].

1.2.1.3 Inhibition of Cyclooxygenase (COX)

COX is the first dedicated enzyme in prostaglandin synthesis (see fig. 1.2.1.3a) and the inhibition of this enzyme as being the means by which NSAIDs exerted their antiinflammatory activity was first proposed by Vane [1971]. Evidence supporting that this was another of the means by which NSAIDs caused damage to the gastrointestinal

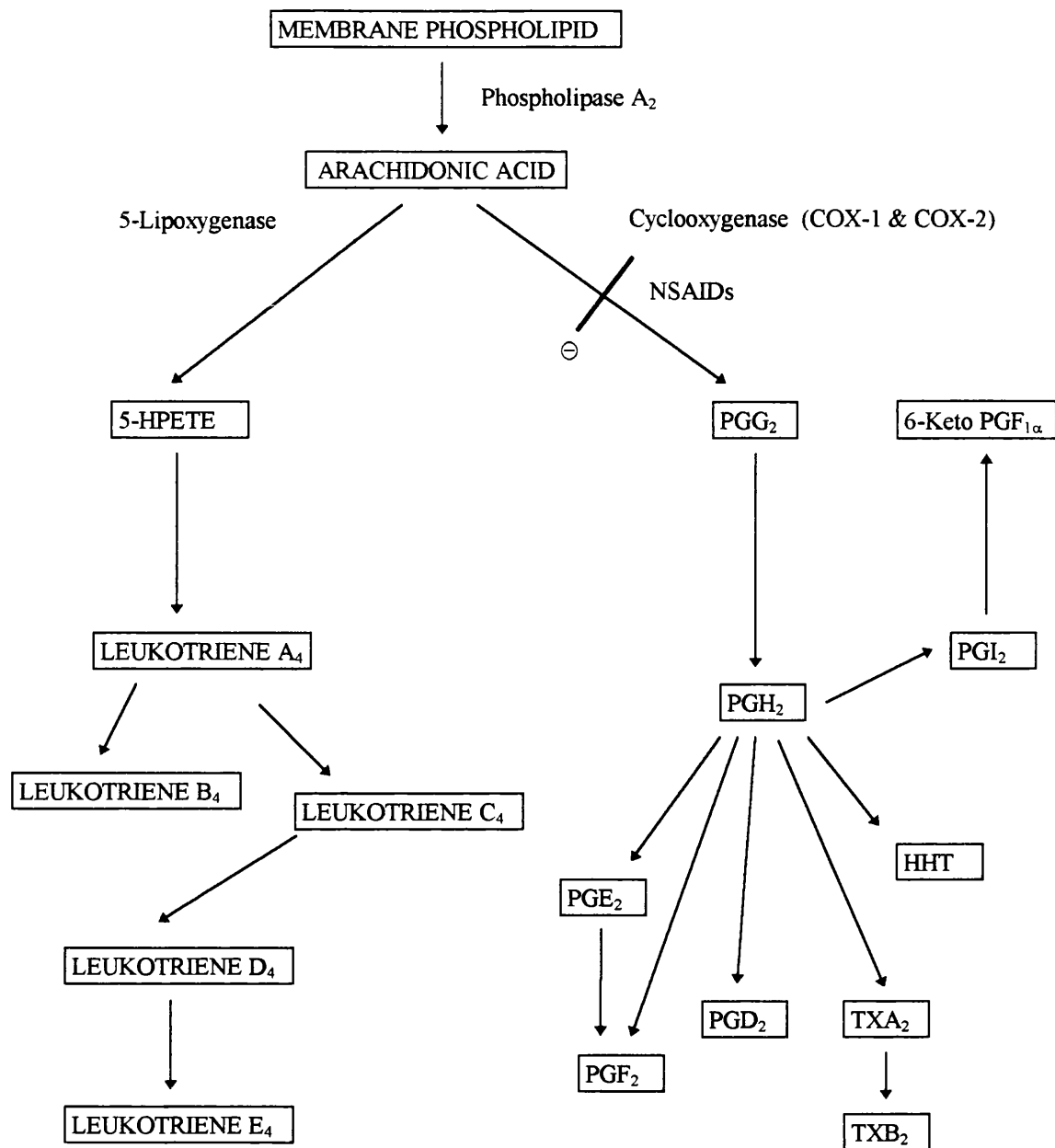


Figure 1.2.1.3a Conversion of Arachidonic Acid to Prostaglandins and Thromboxanes of Series 2 and Leukotrienes of Series 4.

PG	Prostaglandin
PGI	Prostacyclin
HHT	Hydroxyheptadecatrienoate
TX	Thromboxane
5-HPETE	Hydroperoxyeicosatetraenoate

tract was later provided by experiments that showed that a variety of prostaglandins and their analogues could prevent the damage caused by NSAIDs, if given concomitantly [Robert, 1974; Robert, 1975; Fang *et al.*, 1975]. Also drugs, such as prednisolone, which were known to inhibit the formation of arachidonic acid from membrane phospholipid via the enzyme phospholipase A₂ [Gryglewski *et al.*, 1975], were also shown to cause ulceration to the gastrointestinal tract, similar to that found with indomethacin [Lancaster & Robert, 1978]. Thus it was confirmed that prostaglandins conferred a cytoprotective effect [Robert, 1976, 1979] on the gastrointestinal tract but inhibition of their synthesis does not fully explain all mechanisms involved in NSAID-induced damage.

Flower and Vane [1972] proposed that there may be more than one form of COX and that different tissues might contain their own variant of the enzyme. Evidence supporting this theory has been provided by the finding that prostaglandin synthesis could be inhibited by sodium salicylate at inflammatory sites without affecting prostaglandin synthesis in the gastrointestinal tract [Whittle *et al.* 1980]. Conclusive evidence was provided by the characterisation of an isozyme of COX [Xie, *et al.*, 1991] which was different from the one already identified [Yokoyama & Tanabe, 1989] and as a result Vane [1992] has revised his original hypothesis. These two isozymes, which have been designated COX-1 and COX-2, operate in different ways (see fig. 1.2.1.3b).

The COX-1 enzyme is the constitutive form which is responsible for the physiological synthesis of prostaglandins in the gut, kidney, blood platelets [Funk *et al.*, 1991] and other tissues [Simmons *et al.*, 1991]. It is inhibition of this enzyme by NSAIDs which is responsible for the damage they cause to the gastrointestinal tract and the older NSAIDs (indomethacin, naproxen and diclofenac) are more selective for COX-1 than COX-2 [Meade, *et al.*, 1993; Mitchell *et al.*, 1993]. COX-2 is the inducible form which appears to be the major enzyme responsible for inflammatory prostaglandin synthesis and its expression is found to be greatly increased at sites of inflammation [Vane *et al.*, 1994]. In fact, activity of COX-2 is induced by many factors including serum [Simmons, *et al.*, 1991], lipopolysaccharide (LPS) [O'Sullivan *et al.*, 1992a; 1992b] and inflammatory cytokines such as Interleukin-1 [Lyons-Giordano *et al.*, 1992]. One of the newer

NSAIDs, nabumetone, appears to have a higher selectivity for the COX-2 enzyme [Meade, *et al.*, 1993], which may be one of the reasons why it causes very little damage to the gastrointestinal mucosa [Willkins, 1990].

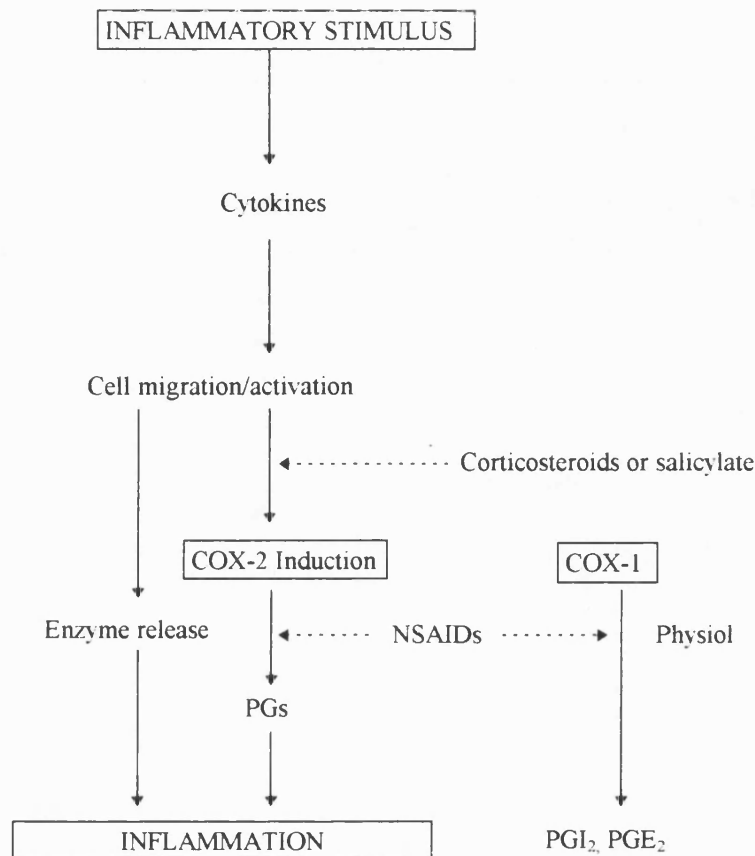


Figure 1.2.1.3b Vane's Revised Hypothesis of the Mechanism of Action of NSAIDs

1.2.1.4 Production of Leukotrienes

It has been suggested that when NSAIDs inhibit COX, the result is that more leukotrienes are formed as 5-lipoxygenase, the first dedicated enzyme in leukotriene synthesis, channels the surplus arachidonic acid down this pathway (see fig. 1.2.1.a) [Rainsford, 1987; Bjarnason *et al.*, 1989]. Leukotrienes have been implicated in the pathogenesis of NSAID-induced damage to the gastrointestinal tract [Rainsford, 1987; Pihan *et al.*, 1988; Asako *et al.*, 1992; Vaananan *et al.*, 1992] and elevated levels of leukotriene B₄ (LTB₄), probably the most important inflammatory leukotriene in man [Lewis & Keft, 1995], have been found in gastric biopsies taken from patients taking

NSAIDs long-term [Hudson *et al.*, 1991]. Leukotrienes stimulate the synthesis of the proinflammatory cytokine IL-1 [Hwang, 1989] and LTB₄ is itself a chemoattractant for neutrophils in humans [Goetzl & Pickett, 1980; Bray *et al.*, 1981; Palmblad *et al.*, 1981; Grisham & Granger, 1988]. The presence of LTB₄ also rapidly increases the cell surface expression of the adhesion glycoprotein CD11/CD18 in activated neutrophils [Zimmerman & McIntyre, 1988; Harlan & Liu, 1992] thus stimulating the adherence of neutrophils to the vascular endothelium [Dahlen *et al.*, 1981; To *et al.*, 1990; Asako *et al.*, 1992]. LTB₄, however, is not thought to be chemotactic for rat neutrophils [Kreisle *et al.*, 1985]. NSAID-induced damage of the gastrointestinal tract has been ameliorated by administering either anti-LTB₄ [Wallace *et al.*, 1992] or an LTB₄ receptor agonist [Asako *et al.*, 1992; Wallace & Granger, 1992; Jacobson, 1995] and also by the direct inhibition of leukotriene synthesis [Samuelson, 1980; Batt, 1992; Musser & Kreft, 1992; Vaananan *et al.*, 1992], however, this protection may have been due to a non-specific action such as free radical scavenging [Rainsford, 1987; Pihan *et al.*, 1988; Vaananan *et al.*, 1992].

1.2.1.5 Adherence and Activation of Neutrophils

Damage to the gastrointestinal tract as a result of neutrophil adherence could occur in one of two ways [Wallace & Granger, 1992]. The signals which cause neutrophils to adhere may also be the ones which activate them, resulting in the release of oxygen derived free radicals and proteases, thus causing injury to the surrounding cells of the epithelium or endothelium. Although all cells possess numerous antioxidant enzymes and scavengers with which to protect themselves from these injurious agents, the rate of production of reactive oxygen metabolites (ROMS) may overwhelm the capacity of the antioxidant defences [Grisham & Granger, 1988] and the imbalance between prooxidative and antioxidative processes, termed 'oxidative stress' [Sies, 1985], results in substantial damage to surrounding tissues. Evidence that free radicals, released from neutrophils after NSAID administration, contribute to gastrointestinal mucosa damage has been shown in rats [Del Soldato *et al.*, 1985; Pihan *et al.*, 1987; Vaananan *et al.*, 1991]. The other way in which neutrophil adherence could result in damage to the gastrointestinal tract is by their obstructing the capillaries in the gastrointestinal mucosa, thus reducing the mucosal blood flow and compromising the defences of the mucosa

accordingly. NSAIDs are known to reduce gastric blood flow [Ashley *et al.*, 1985; Kitahora & Guth, 1987; Gana *et al.*, 1987] and this has been shown to occur after the appearance of white thrombi in the gastric microcirculation [Kitahora *et al.*, 1987]. It has also been demonstrated that NSAID-induced damage to the gastrointestinal tract is significantly reduced in rats which have been depleted of neutrophils, either by methotrexate or anti-neutrophil serum, prior to NSAID administration [Wallace *et al.*, 1990; Lee *et al.*, 1992]. Similarly prevention of neutrophil adherence to the endothelium by an antiserum directed against the neutrophil adhesion glycoprotein CD18 also reduces indomethacin-induced ulceration [Vedder *et al.*, 1988; Wallace *et al.*, 1991].

1.2.1.6 NSAID Uncoupling of Mitochondrial Oxidative Phosphorylation

The uncoupling of oxidative phosphorylation has been implicated as having a role in NSAID-induced damage of the gastrointestinal tract. Earlier experiments show that NSAIDs (salicylate and acetylsalicylate) can act as uncouplers of oxidative phosphorylation [Brody, 1956; Penniall, 1958; Packer *et al.*, 1959; Jeffrey & Smith, 1959; Whitehouse, 1964; Smith & Dawkins, 1971; Tokumitsu *et al.*, 1977; Kawai *et al.*, 1985] and that *in vitro* they can also inhibit a wide range of cellular enzymes such as dehydrogenases [Smith & Dawkins, 1971]. The uncoupling of mitochondrial oxidative phosphorylation and inhibition of mitochondrial dehydrogenases by salicylate are consistent with it having a role in the mitochondrial injury seen in Reye's syndrome [Martens & Lee, 1984].

As a result it has been proposed that NSAIDs, such as indomethacin, cause the uncoupling of mitochondrial oxidative phosphorylation through a topical action as they pass through the gastrointestinal tract, thus disrupting the energy metabolism of the mitochondria in the epithelial cells. Recently, ingestion of NSAIDs has been shown, *in vivo*, to increase the activity of specific mitochondrial and brush border marker enzymes in rats, which is consistent with the uncoupling of oxidative phosphorylation, prior to any visible signs of macroscopic damage to the gastrointestinal mucosa [Bjarnason & Hayllar, 1996]. These events have been mirrored by visible changes in mitochondria viewed via electron microscopy [Bjarnason & Hayllar, 1996]. *In vitro* experiments on isolated coupled rat liver mitochondrial preparations have also shown that all acidic

NSAIDs uncouple mitochondrial oxidative phosphorylation to a similar degree and that their uncoupling potency is correlated to pKa value [Mahmud *et al.*, 1996a, 1996b]. These *in vitro* effects on mitochondrial respiration were found to occur with the NSAID concentrations (μM) at a level easily achievable within the intestinal epithelium [Bjarnason & Hayllar, 1996], and experimental animals with bile duct ligation showed that unmetabolised indomethacin within the gastrointestinal lumen was necessary for the uncoupling to occur [Bjarnason & Hayllar, 1996].

Therefore the pathogenesis of NSAID-induced gastrointestinal damage may be due to uncoupling of mitochondrial oxidative phosphorylation [Hayllar *et al.*, 1991; Somasundaram *et al.*, 1992; Somasundaram *et al.*, 1995; Mahmud *et al.*, 1996a] which depletes the epithelial cells of ATP [Whitehouse, 1964; Mehlman *et al.*, 1972; Tokumitsu *et al.*, 1977; Kawai *et al.*, 1985] resulting in a disruption of epithelial cell junctions [Meza *et al.*, 1980; Madara & Dharmasathaphorn, 1985; Madara *et al.*, 1986, 1987]. The corresponding increase in mucosal permeability [Bjarnason *et al.*, 1993] thus renders the gastrointestinal mucosa exposed to luminal aggressive factors [Somasundaram *et al.*, 1995]. It has therefore been concluded that NSAIDs cause biochemical cell damage, which is quite distinct and severe, resulting in increased intestinal permeability which may in itself be the central mechanism for an inflammatory tissue reaction [Bjarnason & Peters, 1996].

1.2.1.7 Inhibition of Glycolysis and Tricarboxylic Acid Cycle

It has also been proposed that NSAIDs cause damage to the gastrointestinal tract by inhibiting glycolysis and the tricarboxylic acid cycle. This suggestion has been a result of *in vitro* experiments which show that NSAIDs affect glycolysis and also inhibit a wide range of cellular enzymes such as dehydrogenases [Smith & Dawkins, 1971] as well as uncoupling oxidative phosphorylation at a number of sites [Smith & Dawkins, 1971; Mehlman *et al.*, 1971; Glarborg-Jorgensen *et al.*, 1976a; Spenney & Brown, 1977; Tokumitsu *et al.*, 1977; Martens & Lee, 1984; Rainsford, 1989]. (Mitochondrial dehydrogenases are important enzymes in the tricarboxylic acid cycle.) This hypothesis is supported by the fact that a glucose-citrate formulation of indomethacin prevents NSAID-induced gastrointestinal damage in the rat [Rainsford & Whitehouse, 1980].

The mechanism is uncertain but it is thought that NSAIDs may be competitive inhibitors of specific enzymes involved in glycolysis or in the tricarboxylic acid cycle thus causing a reduction in ATP production [Glarborg-Jorgensen *et al.*, 1976a, 1976b; Rainsford & Whitehouse, 1980; Rainsford, 1989] resulting in cell death. An alternative explanation of these findings is that citrate, a potent inactivator of phosphofructokinase, the rate-limiting enzyme of glycolysis, may funnel glucose into the hexose monophosphate pathway and thus provide increased reduction power to combat the oxygen free radicals formed as a result of NSAID metabolism [Bjarnason *et al.*, 1992a]. Thus the concomitant administration of glucose and citrate is thought to provide the epithelial cells of the gastrointestinal tract with the substrates required for the relevant pathways which have been disrupted by the NSAID and as a result prevent the disruption of intercellular junctions [Bjarnason *et al.*, 1992a].

1.2.2 Bacteria of the Upper Gastrointestinal Tract

Initially, due to the hostile environment, the stomach and small bowel were assumed to be sterile [Gorbach *et al.*, 1967]. The stomach because it is bathed in hydrochloric acid at a pH of 1-2, and the small bowel similarly so as the duodenum is regularly flushed with acid (when the stomach empties) and because the jejunum and ileum are regularly in contact with bile salts. As a result the general dogma had been that these environs were free of any colonising bacteria but this was proved to be untrue and the gastrointestinal tract, of any one individual, is now known to host more than 400 bacterial species, some of them pathogens [Simon & Gorbach, 1986, 1987].

The microflora of the stomach is mostly gram-positive and aerobic (commonly streptococci, staphylococci and lactobacilli) [Gorbach *et al.*, 1967] whereas the distal ileum predominantly contains anaerobes such as *Bacteroides*, *Bifidobacterium*, *Fusobacterium* (which are gram-negative) and *Clostridium* (which is gram-positive) [Gorbach *et al.*, 1967; Draser *et al.*, 1969; Drasar & Shiner, 1969; Simon & Gorbach, 1984]. Stretching between these two extremes, the sparsely populated stomach and the more densely populated distal ileum, the rest of the small bowel contains a mixture of gram-negative and gram-positive bacteria at varying densities depending on their location.

1.2.2.1 *Helicobacter pylori*

Helicobacter pylori is a gram-negative bacterium with a curved, spiral or gull-wing shape. It is 2.5 to 3.5 μm long, 0.5 to 1.0 μm in diameter and has a periodicity of 1.0 to 2.0 μm . The surfaces of this bacterium are smooth and one to six flagellae (which are sheathed and contain a terminal bulb) emerge from one of its rounded ends. The flagellae, which are thought to be sheathed in order to protect them from the acidic environment, are necessary for motility but are not a prerequisite for attachment to gastric cells [Eaton *et al.*, 1989, 1991]. Initially, owing to its close similarity to *Campylobacter jejuni*, it was called *Campylobacter pylori* but it has since been reclassified and now is universally known as *Helicobacter pylori*. [Dubois, 1995].

Helicobacter pylori, a type I carcinogen [Logan, 1994], colonises the non-acid secreting mucosa of the stomach and is seldom found in the area where parietal cells are numerous. As a result it is found in the gastric antrum, cardia, corpus and attached to the gastric epithelial cells of the duodenum. (The tissue specific binding of *Helicobacter pylori* has been demonstrated *in vitro* since binding of the bacteria is significantly increased in human gastric cells as opposed to non-gastric ones [Nilius *et al.*, 1993; Smoot *et al.*, 1993].) *Helicobacter pylori* multiplies rapidly within the stomach but not in the lumen because it prefers to live within the pH range 4 to 7 [Dubois, 1995].

Helicobacter pylori was first linked to gastritis and gastric and duodenal ulceration by Marshall and Warren [1983, 1984] but almost 15 years later the mechanisms involved are still not truly understood. Initially it was thought that the bacterium's potent urease, which it uses to convert urea into ammonia [Cover *et al.*, 1991; el Nujumi *et al.*, 1992], thus making its environment more habitable [Perez-Perez *et al.* 1992; Segal *et al.*, 1996]; was responsible for all the inflammation seen. Although ammonia generated by the urease has been shown to be a potent cytotoxin for many cell lines *in vitro* [Xu *et al.*, 1990; Megraud *et al.*, 1992], particularly gastric ones [Smoot *et al.*, 1990; Segal *et al.*, 1996], and *in vivo* [Eaton *et al.*, 1991] this is not now thought to be the major culprit.

There are known to be two broad types of the bacterium, classified as type 1 and type 2, and it is the type I bacteria which have been found to be the most virulent strains [Xiang

et al., 1995]. Type 1 possess a larger DNA region [Segal, 1997] which contain the genes, *vacA* and *cagA*, that enable these bacteria to produce vacuolating cytotoxin and cytotoxin-associated protein, respectively. It is the type I strains that are implicated in the pathogenesis of duodenitis, duodenal ulcers and gastric cancer [Covacci *et al.*, 1993; Xiang *et al.*, 1995; Weel *et al.*, 1996] although not all humans infected with these strains go on to develop a duodenal ulcer.

Helicobacter pylori-positive patients with duodenal ulcer have been found to have higher fasting serum gastrin levels than those who only have gastritis and they in turn have fasting serum gastrin levels higher than the negative controls [Haruma *et al.*, 1995]. Gastric acid secretion is higher in patients with duodenal ulcer than those without [Blair *et al.*, 1987; Feldman *et al.*, 1988] and it has been suggested that *Helicobacter pylori* infection increases gastric acid secretion through hypergastrinaemia [Levi *et al.*, 1989a] owing to the trophic effects on parietal cells [Johnson, 1976]. However, recent studies have reported that although eradication of *Helicobacter pylori* consistently lowers serum gastrin levels [Marshall *et al.*, 1988; Levi *et al.*, 1989b; Graham *et al.*, 1990] this has had no corresponding effect on basal or maximal acid output [Montbriand *et al.*, 1989] or on intragastric pH [McColl *et al.*, 1991].

Studies on the regulation of gastrin secretion show that gastrin G cells are under constant restraint by somatostatin D cells in the antral mucosa [Saffouri *et al.*, 1980; Schubert *et al.*, 1988] and the presence of high gastric acid secretion increases somatostatin release resulting in reduced gastrin secretion. It has been proposed that the modification of the gastric surface pH via neutralisation of gastric acid by ammonia [Levi *et al.*, 1989b], produced through the urease activity of *Helicobacter pylori*, results in lower antral somatostatin concentration [Kaneko *et al.*, 1992]. Thus enhanced gastrin synthesis and release occur as a result of the inhibition of somatostatin synthesis in spite of the low intraluminal pH [Queiroz *et al.*, 1993].

More than 95 per cent of patients with duodenal ulcer and more than 80 per cent of patients with gastric ulcer are infected with *Helicobacter pylori* [Peterson, 1991; Graham, 1991; Veldhuyzen van Zanten *et al.*, 1994] but only 15 to 20 per cent of those

infected will develop an ulcer during their lifetime [Sipponen *et al.*, 1989] so *Helicobacter pylori* does not appear to be the sole causative agent. Other factors which increase the risk of *Helicobacter pylori*-positive subjects developing an ulcer include cigarette smoking [van Deventer *et al.*, 1989] and the taking of NSAIDs [Jones *et al.*, 1991]. However, in the majority of cases, *Helicobacter pylori* infection, once diagnosed, can be easily eradicated by a combination of two antibiotics, amoxycillin [Adamek *et al.*, 1993] and metronidazole [Rautelin *et al.*, 1992], in conjunction with an antimicrobial agent such as bismuth (which disrupts the integrity of bacterial cell walls [van Caekenberghe & Breysens, 1987]) [Walsh & Peterson, 1995].

1.2.2.2 Bacteria of the Rat Small Bowel

Bacteria have been shown to be mediators in NSAID-induced damage to the gastrointestinal mucosa in the rat small bowel [Kent *et al.*, 1969]. After administration of NSAIDs the numbers of bacteria in the rat small bowel are seen to increase along with the formation of ulcers [Kent *et al.*, 1969; Yamada *et al.*, 1993]. This ulceration is prevented or reduced by concomitant or pre-treatment with various antibiotics [Kent *et al.*, 1969; Fang *et al.*, 1977; Yamada *et al.*, 1993; Collins *et al.*, 1995]. A similar result has been obtained using germ-free rats [Robert & Asano, 1977] which, when mono-contaminated with *Escherichia coli*, developed ulcers in the small bowel although these were less severe than those found in germ-free rats contaminated with the normal microflora of rat small bowel [Robert & Asano, 1977]. These results suggest that it is the antimicrobial action of the antibiotics which attenuate the formation of NSAID-induced ulceration. However, some researchers believe it is a direct effect of the antibiotics themselves on the cells of the gastrointestinal mucosa which is cytoprotective [Satoh, *et al.*, 1983] as some antibiotics have been found to control the production of IL-2 *in vitro* [Braquet & Rola-Pleszczynski, 1987]. Metronidazole has been reported as being an immunosuppressant [Grove *et al.*, 1977] possibly by acting as an inhibitor of leucocyte-endothelial cell adhesion [Arndt *et al.*, 1994] or as a scavenger for free radicals [Akamatsu *et al.*, 1990].

1.2.3 Contributory Mechanisms of Ulcerogenesis

Although the mechanisms associated with the following environmental factors were not investigated during the course of this research project, they are believed to exacerbate the damage caused by other pathogens such as NSAIDs and *Helicobacter pylori*.

1.2.3.1 Smoking

Cigarette smoking has long been implicated as having a role in ulcer formation as people who smoke are at a greater risk of developing an ulcer than non smokers [Friedman *et al.*, 1974; Piper *et al.*, 1982; Kato *et al.*, 1992] and these ulcers are more likely to be in the stomach rather than in the duodenum [Friedman *et al.*, 1974]. In addition, ulcers take longer to heal in patients who smoke [Doll *et al.*, 1958; Chujoh & Nakazawa, 1981; Korman *et al.*, 1981, 1982; Massarrat & Eisenmann, 1981] and are also more likely to recur [Sontag *et al.*, 1984; Korman *et al.*, 1983]. Inhalation of cigarette smoke alone, however, does not appear to cause gastrointestinal ulceration [Toon *et al.*, 1983] so it would appear that cigarette smoking mediates mucosal damage which is being caused by some other noxious agent. Various studies have shown that cigarette smoking not only mediates mucosal damage it also attenuates mucosal protection.

1.2.3.1.1 Smoking as a Mediator of Mucosal Damage

The major cause of gastric ulceration is thought to be excessive levels of gastric acid secretion [Koelz *et al.*, 1986] and smokers are found to have increased basal and peak gastric acid output [Steigmann *et al.*, 1954; Piper & Raine, 1959; Murthy *et al.*, 1977; Sontag *et al.*, 1984; Parente *et al.*, 1985; Whitfield & Hobsley, 1987; Lanas & Hirschowitz, 1992]. Cigarette smoking also enhances the rate of nocturnal secretion of acid [Weir, 1988] and reverses the inhibition of nocturnal acid secretion produced by H₂ receptor antagonists [Boyd *et al.*, 1983].

Pepsin is an aggressive factor to the gastrointestinal mucosa [Shay, 1961] and smokers have been found to have significantly higher basal, vagally stimulated and maximal levels of pepsin with or without duodenal ulcer [Lanas & Hirschowitz, 1992]. It has also been reported that after administration of histamine or pentagastrin to patients with peptic

ulcer, pepsin secretion was higher in smokers than non smokers [Walker & Taylor, 1979].

Reflux of bile salts into the stomach causes damage to the gastric mucosa [Rhodes *et al.*, 1969] and long term smokers have been shown to have an increased susceptibility to duodenogastric reflux [Dippy *et al.*, 1973; Read & Grech, 1973; Müller-Lissner, 1986].

Helicobacter pylori are known mediators of gastrointestinal ulceration and cigarette smoking increases the risk of infection [Bateson, 1993]. It has also been found that nicotine increases the activity of the vacuolating toxin associated with the more virulent strains of *Helicobacter pylori* [Cover *et al.*, 1992].

Oxygen-derived free radicals are known to mediate damage to the gastrointestinal mucosa and levels of neutrophils actively producing these free radicals are found to be higher in the blood of smokers [Kalra *et al.*, 1991].

Patients taking NSAIDs are at risk of developing gastrointestinal ulcers and cigarette smoking increases the risk of bleeding in the upper gastrointestinal tract of these patients by 40 per cent [Rodriguez & Jick, 1994].

1.2.3.1.2 Smoking as an Attenuator of Mucosal Defence

The integrity of the gastrointestinal mucosa is dependent on the cytoprotective effect of prostaglandins and their production is reduced in smokers [Balint & Varró, 1986; Quimby *et al.*, 1986; Hudson *et al.*, 1990; Cryer *et al.*, 1992]. Prostaglandins enhance the mucosal defence systems, by inhibiting the secretion of gastric acid [Weir, 1988], stimulating the output of bicarbonate [Bolten & Cohen, 1978; Kauffman *et al.*, 1980] and mucus [Soll *et al.*, 1989; Bickel & Kauffman, 1981; McQueen *et al.*, 1983], and, in addition to their protective properties on a cellular level [Tarnawski *et al.*, 1988], help to maintain the mucosal blood flow [Main & Whittle, 1973; Gerber & Neis, 1982; Silen, 1987; Whittle & Vane, 1987]. Prostaglandins also inhibit free radical production and enzyme release by neutrophils [Gryglewski *et al.*, 1987; Kainoh *et al.*, 1990] and also stabilise mucosal mast cells [Bickel & Kauffman, 1981; McQueen *et al.*, 1983].

Cigarette smoking decreases the gastrointestinal blood flow [Fletcher *et al.*, 1985; Kawano *et al.*, 1989a, 1989b], which is believed to compromise the defences of the mucosa, and blood flow is also known to be reduced at sites of ulceration [Ashley *et al.*, 1985]. Blood flow correlates well with rates of bicarbonate secretion [Nagata & Guth, 1983] which is itself elevated in response to histamine. Histamine is released during injury to the gastrointestinal mucosa [Fromm, 1987], which occurs continuously as a result of food and other trauma [Soll *et al.*, 1989], and the ability of the blood to provide a buffer to the mucosa and thus protect the damaged area from the low intraluminal pH is decreased by the reduction in blood flow.

Epidermal growth factor (EGF) is secreted into the saliva and as a result enters the stomach [Gregory *et al.*, 1979]. It has been reported to have a cytoprotective effect [Endoh & Leung, 1994], one of which is its ability to inhibit gastric acid secretion [Elder *et al.*, 1975]. Smoking one cigarette every 30 minutes has been found to lower basal-salivary EGF secretion in healthy human subjects [Konturek *et al.*, 1989] and this has been linked to an increased risk of gastric ulceration [Ohmura *et al.*, 1987; Jones *et al.*, 1992].

It has been proposed that the development of a gastrointestinal ulcer depends on the balance between protective and aggressive factors which results in an ulcer when the balance is tipped in favour of the aggressors [Shay, 1961]. Thus it would appear that although inhalation of cigarette smoke alone does not directly lead to gastrointestinal ulceration, coupled with any of the aggressive factors it is able to tip the balance towards ulcer formation.

1.2.3.2 Alcohol

Alcohol has been implicated as being a risk factor for gastric ulceration and patients taking NSAIDs who are heavy drinkers of alcohol have an increased risk of upper gastrointestinal bleeding [Carson *et al.*, 1987]. Topical application of 60 per cent (v/v) ethanol to the gastric mucosa of rats has been shown to induce an immediate leakage of macromolecules, such as albumin, out of capillaries and the postcapillary venules into the interstitial space [Szabo *et al.*, 1985; Kalia *et al.*, 1997]. This leakage occurs primarily in

regions of the gastric mucosa where there has been an accompanying decrease in blood flow [Szabo *et al.*, 1985; Kalia *et al.*, 1997] and is indicative of damage to the endothelium [Szabo *et al.*, 1985; Kalia *et al.*, 1997]. Studies of these areas, on a cellular level, show that whereas no circulating or adherent leucocytes are visible in the microvasculature of the control gastric mucosa, shortly after ethanol administration a number of adherent leucocytes begin to appear on the edge of the area of blood flow stasis [Kalia *et al.*, 1997]. It has been proposed that a close association exists between reduction in blood flow and damage to the gastrointestinal mucosa [Ashley, *et al.*, 1985; Pihan *et al.*, 1986; Bou-Abboud *et al.*, 1988] and that some areas of the mucosa are more susceptible to ischaemia and its related injury than others [Piasecki, 1991]. This would explain the fact that although noxious substances such as ethanol bathe the entire area of the gastric mucosa to a similar extent, lesions only occur at a few focal points.

Studies have demonstrated that a change in blood flow occurs prior to ulcer formation [Szabo *et al.*, 1985; Pihan *et al.*, 1986; Bou-Abboud *et al.*, 1988;] and evidence exists that the areas of gastric ulceration are linked closely to the microvasculature [Anthony *et al.*, 1995, 1996]. One hypothesis is that certain regions of the gastric mucosa may be more susceptible to ulceration than others owing to the presence of end arteries [Piasecki, 1991]. Studies that have shown that there is a link between vascular stasis and mucosal necrosis [Szabo *et al.*, 1985; Pihan *et al.*, 1986; Bou-Abboud *et al.*, 1988;] are supported by reports detailing neutrophils as being mediators in the formation of ethanol-induced ulcers [Kvietys *et al.*, 1989, 1990;]. Thus it would appear that the initial damage to the endothelium caused by the topical effect of the ethanol [Pihan *et al.*, 1986], visible as gaps between the endothelial cells when viewed via electron microscopy [Kalia *et al.*, 1997]; is exacerbated by the neutrophil response [Grisham & Granger, 1988] and the resultant tissue damage [Weiss, 1989; Kvietys *et al.*, 1990].

1.2.3.3 Stress

Controversy surrounds the subject of stress ulcers [Konturek *et al.*, 1990], first described 60 years ago [Selye, 1936], as some researchers disagree with the premise that emotional stress can lead to ulceration of the gastrointestinal mucosa owing to a lack of evidence supporting this theory [Piper *et al.*, 1978, 1981; Thomas & Piper, 1980]. Although

emotional stress alone is unlikely to result in gastrointestinal ulceration it is thought by some that, in conjunction with other risk factors, emotional stress may mediate damage to the gastric mucosa [Spicer *et al.*, 1944; Weiner *et al.*, 1957; Brodie & Hanson, 1960; Desiderato *et al.*, 1974; Peters & Richardson, 1983; Walker *et al.*, 1988]. It has been suggested that people experiencing acute emotional stress are likely to smoke more cigarettes and have an increased alcohol consumption than unstressed individuals who share the habit and these two practices have been implicated as exposing patients to a higher risk of gastrointestinal ulceration [Friedman *et al.*, 1974; Cummings *et al.*, 1983; Ostensen *et al.*, 1985; Ganz, 1987]. There is also a possibility that chronic emotional stress in humans may have an effect on the vagus nerve, resulting in its chronic stimulation which is known to lead to an increase in parietal cell density and chief cell density with an associated rise in gastric acid secretion and hyperpepsinogenaemia [Pearl *et al.*, 1965, 1966; Thirlby & Feldman, 1984].

Physiological stress, however, as a result of trauma through burns [Czaja *et al.*, 1974], shock, sepsis [Moody *et al.*, 1976] or serious injury [Lucas *et al.*, 1972] particularly to the head [Watts & Clark, 1969; Norton *et al.*, 1970], is accepted as being a cause of damage to the gastrointestinal mucosa [Stremple *et al.*, 1973, Robert & Kauffman, 1983]. Even so, most patients exposed to severe physiological stress do not go on to develop erosions or ulcers to the gastrointestinal mucosa and in one study only 59 cases of gastric erosions were found out of 2,600 post mortems carried out [Moody *et al.*, 1976]. Patients who do present with upper gastrointestinal bleeding after head injury or neurosurgery are therefore unusual [Watts & Clark, 1969; Norton *et al.*, 1970] and in this group the erosive gastritis is associated with hypersecretion of gastric acid.

Experiments with animals indicate that acute stress inhibits gastric epithelial proliferation rates [Kim *et al.*, 1967; Lahtiharju & Rytomaa, 1967; Imondi *et al.*, 1968; Ludwig & Lipkin 1969; Willems & Lehy, 1975; Takeuchi & Johnson, 1979; Kuwayama & Eastwood, 1985] and increases the rate of surface cell loss in the fundus whereas it has no effect on epithelial proliferation in the antrum [Kuwayama & Eastwood, 1985]. This finding correlates with the fact that ulcers induced by physiological stress tend to appear in the mucosa of the fundus rather than the antral mucosa [Kuwayama & Eastwood,

1985]. The gastric epithelium needs to be constantly replaced, as epithelial cells regularly slough off naturally and the mechanics of ingestion continually cause damage to the gastric mucosa [Eastwood, 1977]. Consequently any reduction in the replacement rate of worn out or damaged epithelial cells leaves the gastric mucosa with an area susceptible to attack from acid in the gastric lumen. Restraint stress in animals also causes a reduction in blood flow [Guth, 1972] within the fundic mucosa which probably is responsible for the reduced rate of proliferation in this region.

1.3 CYTOKINES AND THEIR ROLE IN THE INFLAMMATORY PROCESS

Cytokines are a family of small host-derived proteins or glycoproteins produced by cells involved in the host's immune response to injury, invasion, infection and inflammation [Tracey & Cerami, 1993]. They act locally as messengers between cells and are mediators in the regulation of the immune response [Kroemer & Martinez, 1991] controlling the production and effect of other cytokines (see fig. 1.3).

1.3.1 Tumour Necrosis Factor α (TNF- α)

TNF- α is the major cytokine involved in the host response to bacterial invasion [Fong *et al.*, 1989], responding to the bacterium itself as well as to various bacterial toxins, including endotoxins, such as lipopolysaccharide, a large molecule in the outer cell walls of gram-negative bacteria; and exotoxins produced by gram-positive bacteria such as *Staphylococci* and *Streptococci* [Dinarello, 1991]. Production of TNF- α initiates the inflammatory cytokine cascade [Fong *et al.*, 1989; Tracey & Cerami, 1993] by stimulating neighbouring leucocytes and endothelial cells to synthesise and secrete IL-1. Production of TNF- α can also be stimulated by itself and by other cytokines such as IL-1, IL-2 and granulocyte-macrophage-colony stimulating factor (GM-CSF) [Tracey, 1994]. TNF- α is a potent stimulator of various immune responses but in the presence of IL-1 these responses are more than doubled as TNF- α and IL-1 together amplify their effects synergistically [Elias *et al.*, 1987; Mandrup-Poulsen, *et al.*, 1987; Okusawa *et al.* 1988]. (This synergism appears to be caused by second messenger molecules rather than by up regulating cell receptors as IL-1 reduces the surface expression of TNF receptors

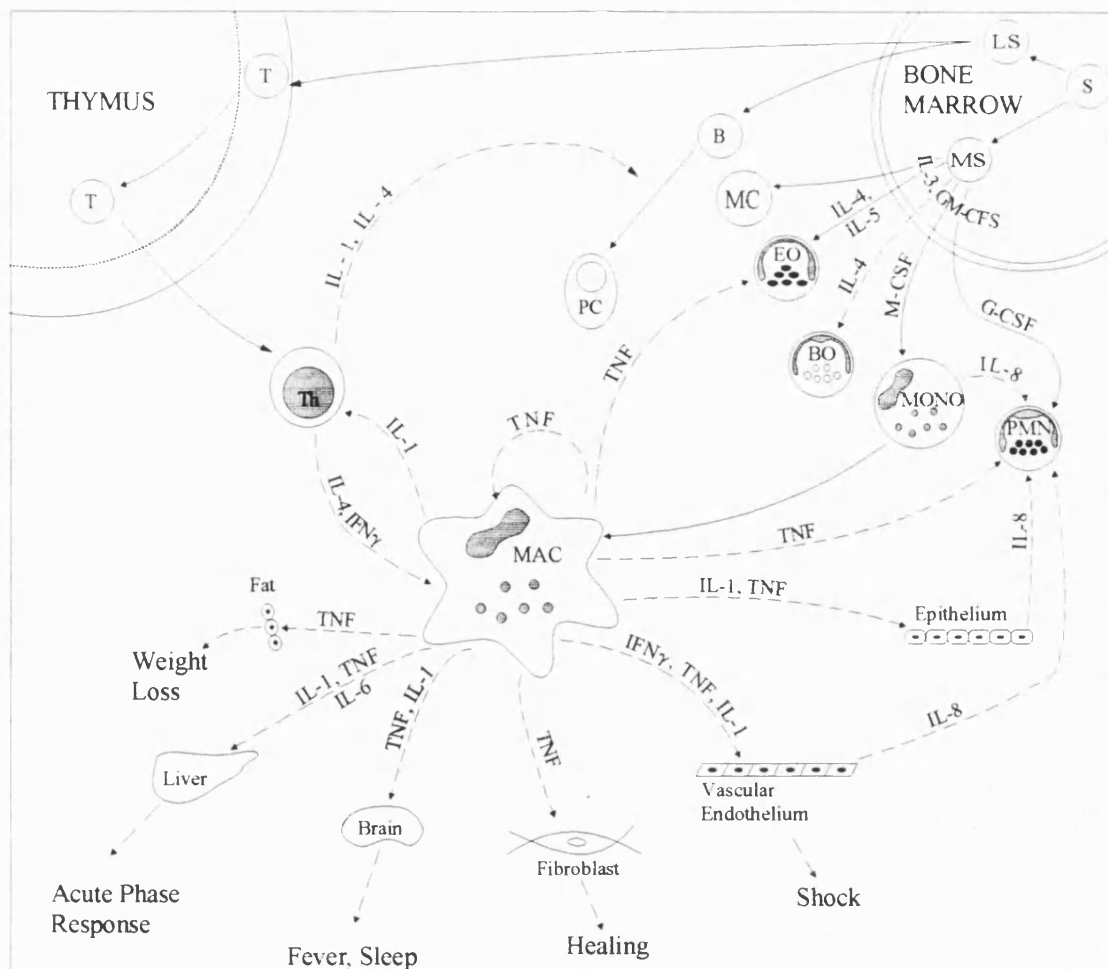


Figure 1.3 Interrelationship of Cytokines Studied During this Research

S	Stem Cell
MS	Marrow Stem Cell
LS	Lymphoid Stem Cell
B	Bone Marrow Derived Lymphocyte
PC	Plasma Cell
T	Thymus Derived Lymphocyte
Th	T Helper Cell
MAC	Macrophage
MONO	Monocyte
PMN	Polymorphonuclear Leucocyte (Neutrophil)
EO	Eosinophil
BO	Basophil
MC	Mast Cell
GM-CSF	Granulocyte-Macrophage-Colony Stimulating Factor
G-CFS	Granulocyte-Colony Stimulating Factor
M-CSF	Macrophage-Colony Stimulating Factor

[Holtmann & Wallach, 1987; Wallach *et al.*, 1989].) These mediators in turn stimulate surrounding tissue cells, such as the gastric epithelial cells and gastric endothelial cells, to secrete IL-8, a potent chemoattractant for neutrophils which causes migration of neutrophils to the infected area and primes them to release their destructive contents against the invading organisms.

Human TNF- α is produced principally by macrophages and monocytes but is also made by other cells including basophils, eosinophils, natural killer cells, mast cells, B cells and T cells [Gordon & Galli, 1991, 1994; Galli, 1993; Tracey & Cerami, 1993; Tracey, 1994]. Initially TNF- α is synthesised as a large precursor molecule, 233 amino acids long [Wang *et al.*, 1985; Spies *et al.*, 1986; Davis *et al.*, 1987; Muller *et al.*, 1987], that is cleaved by proteases into the smaller active form, 17 kilodaltons in size and made up of 157 amino acids, which is secreted outside the cell and is the biologically active form [Tracey & Cerami, 1993]. There is, however, an intermediate membrane associated form, 26 kilodaltons in size, which is also biologically active [Kriegler *et al.*, 1988; Jue *et al.*, 1990; Perez *et al.*, 1990]. TNF- α causes fever [Dinarello, 1986; Dinarello *et al.*, 1986a, 1986b], anorexia, shock [Okusawa *et al.*, 1988], hypotension and capillary leakage as well as increasing skeletal muscle degradation and lipolysis [Dewhirst *et al.*, 1985; Krane *et al.*, 1985; Warren *et al.*, 1987; Starnes *et al.*, 1988; Spriggs, *et al.*, 1988; Bauer *et al.*, 1989].

1.3.2 Interleukin 1 (IL-1)

There are two forms of IL-1, IL-1 α and IL-1 β , which are the products of different genes and as a result have differing amino acid sequences. Nevertheless, despite these differences, their three dimensional structure is similar enabling them to act through the same receptors and as a result share the same biological activities [Dinarello, 1991]. Even so IL-1 α has a greater affinity for the Type I receptor, which is found on most cells, and IL-1 β has a greater affinity for the Type II receptor, found primarily on neutrophils, monocytes, bone marrow cells and B lymphocytes [Dinarello & Wolff, 1993]. Both proteins are rapidly synthesised, primarily by monocytes and macrophages, in response to other cytokines such as TNF- α [Dinarello *et al.*, 1986b; Dinarello & Wolff, 1993], granulocyte-macrophage-colony stimulating factor (GM-CSF) [Sisson &

Dinarello, 1988], macrophage-colony stimulating factor (M-CSF) [Dinarello, 1991] and to microbial toxins, inflammatory agents, products of activated lymphocytes, complement and clotting factor [Dinarello, 1991]. Synthesis of IL-1 is not restricted to leucocytes, however, and it is found in nearly all tissue [Dinarello, 1991; Dayer & Fenner, 1992] including epithelial cells and endothelial cells which also secrete IL-1 in response to extracellular TNF- α [Libby *et al.*, 1986; Nawroth *et al.*, 1986; Tracey & Cerami, 1993].

Initially both human forms of the cytokine are synthesised as a large precursor molecule with a molecular weight of around 31 kilodaltons but after enzyme cleavage the mature proteins are about 17.5 kilodaltons in size [Dinarello & Wolff, 1993], IL- α being made up of 159 amino acids and IL-1 β made up of 153 amino acids. The IL- α precursor is active prior to cleavage [Howard *et al.*, 1991] and several common enzymes are able to cleave it into smaller and more active forms. Conversely IL-1 β must be cleaved for it to have optimum biological activity and only one protease, IL-1 β -converting enzyme (ICE), appears to be highly specific in cleaving the 31 kilodalton IL-1 β precursor leaving it in its 17.5 kilodalton active form [Kostura *et al.*, 1989; Schmidt & Tocci, 1990; Cerretti *et al.*, 1992; Thornberry *et al.*, 1992]. During the first 24 hours of activation, after stimulation with bacterial toxins, most IL-1 α (80 per cent) remains within the monocyte/macrophage [Lasfargues *et al.*, 1987; Howard *et al.*, 1991], either free within the cytosol [Lasfargues *et al.*, 1987] or bound to the inner membrane of the cell, although extracellular concentrations of IL-1 α are detectable within 6 hours [Lonnemann *et al.*, 1989]. These extracellular levels gradually increase until they exceed cell-associated levels after 48 hours [Lonnemann *et al.*, 1989]. Conversely the majority of IL-1 β is secreted into the extracellular space, via mechanisms such as exocytosis from vesicles, active transport by multi-drug resistant proteins or by the death of the cell itself [Dinarello & Wolff, 1993], reaching its maximal levels after about 12 hours [Lonnemann *et al.*, 1989]. IL-1 is a stimulus for its own gene expression and synthesis in blood mononuclear cells, fibroblasts, endothelial and smooth muscle cells [Dinarello *et al.*, 1987; Warner *et al.*, 1987a, 1987b] although steady state levels are slower to increase [Schindler *et al.*, 1990]. Endothelial cells exposed to IL-1 produce IL-8, [van Damme, 1994] a potent

chemoattractant for neutrophils, and also produce adhesion molecules which mediate neutrophil adhesion [Dejana *et al.*, 1987].

IL-1 α and IL-1 β both induce fever, sleep, shock and anorexia [Dinarello & Wolff, 1993] and a single intravenous injection decreases mean arterial pressure, lowers systemic vascular resistance and induces leucopenia and thrombocytopenia [Okusawa *et al.*, 1988; Fischer *et al.*, 1991]. IL-1 also stimulates T cells and B cells [Neta *et al.*, 1988].

1.3.3 Interleukin 4 (IL-4)

IL-4 is a type 2 cytokine which means that its role is an antiinflammatory one and thus it is able to down regulate the cellular immune response to infection by inhibiting many of the leucocyte responses [Danzer, *et al.*, 1994; D'Andrea *et al.*, 1995]. IL-4 is thought to contribute to the balance between the production of IL-1 and IL-1 receptor antagonist (IL-1ra) by increasing the formation of IL-1ra [Kam *et al.*, 1995] as well as by decreasing the production of IL-1 itself [Hart *et al.*, 1989; Vannier *et al.*, 1992]. (The protective effect of IL-1ra is thought to be its ability to neutralise the synergistic effect between IL-1 and TNF- α as IL-1 alone is not very toxic [Waage & Espevik, 1988].) This balance is important for whereas small amounts of IL-1 may be necessary to maintain the host's immune defences, large amounts in the presence of TNF- α can be lethal [Waage & Espevik, 1988; Fong *et al.*, 1989; Waage *et al.*, 1989; Munoz *et al.*, 1991; Cannon *et al.*, 1992]. IL-4 is also thought to inhibit the synthesis of TNF- α [Fan *et al.*, 1995] and interferon- γ (IFN- γ), both type 1 cytokines [Vercelli *et al.*, 1990; Erard *et al.*, 1993; Danzer, *et al.*, 1994], and to enhance the production of IL-10, another type 2 cytokine [Danzer, *et al.*, 1994; D'Andrea *et al.*, 1995].

IL-4 is mainly synthesised by activated T cells [Poo *et al.*, 1988], but is also produced by mast cells and basophils [Piccinni *et al.*, 1991; Brunner *et al.*, 1993]. It has a wide range of biological activity on leucocytes, such as macrophages, natural killer cells, B cells and T cells, and also on fibroblasts and endothelial cells [Banchereau & Rybak, 1994]. Initially human IL-4 is synthesised as a precursor molecule, 153 amino acids long [Yokota *et al.*, 1986], that is cleaved by proteases into the smaller biologically active form, 20 kilodaltons in size and made up of 129 amino acids, which is secreted outside the cell [Banchereau & Rybak, 1994].

1.3.4. Interleukin 8 (IL-8)

IL-8 belongs to a group of cytokines called the Chemokines. These are a group of cytokines which are related in sequence and structure. They are all about 8-10 kilodaltons in size and fall into two groups depending on whether the first two of the four cysteine residues, common to all chemokines and involved in the tertiary structure, are adjacent (C-C) or separated (C-X-C) by another amino acid [Oppenheim *et al.*, 1991; Schall, 1991]. Chemokines selectively attract and stimulate distinct leucocyte populations unlike other inflammatory agents such as leukotrienes which are less specific chemoattractants for leucocytes. IL-8 belongs to the C-X-C subgroup and is a potent chemoattractant for neutrophils, directing their migration [Schröder *et al.*, 1987; Yoshimura *et al.*, 1987a; van Damme *et al.*, 1988] to sites of infection and inflammation and also inducing their adhesion to endothelial cells [Carveth *et al.*, 1989] and activation by degranulation [Walz *et al.*, 1987].

IL-8 is synthesised and released by a number of leucocytes including monocytes [Matsushima *et al.*, 1988], macrophages [Walz *et al.*, 1987; Yoshimura *et al.*, 1987b], T cells [Gregory *et al.*, 1988] and neutrophils [Strieter *et al.*, 1990]. However, IL-8 is also produced by cells which play an important role in the mediation of inflammation, immunity and wound healing [Miller & Krangel, 1992] such as fibroblasts [van Damme *et al.*, 1989a, Larsen *et al.*, 1989a; Schröder *et al.*, 1990], endothelial cells [Strieter *et al.*, 1989] and gastric epithelial cells [Crabtree *et al.*, 1994]. These cells produce IL-8 after stimulation by IL-1 or TNF- α [van Damme, 1994] although synthesis can be directly induced in fibroblasts by viruses [van Damme *et al.*, 1989a]. Initially IL-8 is synthesised as a precursor molecule, 99 amino acids long, that is cleaved by proteases into smaller forms, the 8 kilodaltons size, made up of 72 amino acids, being the most biologically active [Lindley *et al.*, 1988, Gimbrone *et al.*, 1989]. From its three dimensional structure, natural IL-8 appears to occur as a doublet [van Damme *et al.*, 1989b] held together by hydrogen bonds.

Rats do not synthesise IL-8 but they do have an equivalent (C-X-C) chemokine protein which is called Cytokine-Induced Neutrophil Chemoattractant (CINC) and as the name suggests operates in a parallel fashion. It is also released from similar cells, in the same

manner as IL-8, after stimulation by IL-1 and TNF- α . [Nakagawa *et al.*, 1993, Zagorski & De Larco, 1993; Blackwell *et al.*, 1994; Harada *et al.*, 1994; Koike *et al.*, 1994; Wu *et al.*, 1994; Aihara *et al.*, 1995].

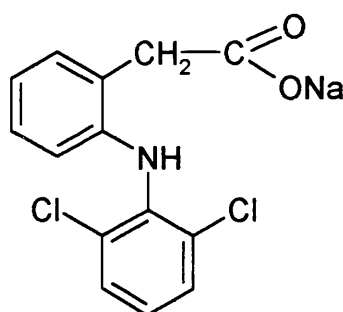
1.4 NSAIDS USED DURING THIS PROJECT

Almost 300,000 prescriptions for NSAIDs are written each year per million inhabitants of the Western world [Walt *et al.*, 1986] and total sales of NSAIDs are in the region of £4 billion annually [Garner, 1992] making this group of drugs the most successful ever developed [Bjarnason *et al.*, 1992b]. Although these drugs are very efficacious they have an unfortunate side effect in that they are known to have a detrimental effect on the mucosa of the gastrointestinal tract. Initially the problem was thought to be mainly restricted to the stomach but during the past 20 years it has become increasingly evident that, during long-term use, they have a more deleterious and serious effect in the duodenum [Hawkey, 1990] and the rest of small bowel [Sturges & Krone, 1973; Schwartz, 1981]. Lesions here are difficult to quantify, they fail to show up during X-ray procedures [Collins *et al.*, 1994] and conventional endoscopes cannot reach far enough into the small bowel to visualise them, however, anaemia, accompanied by blood and protein loss in the faeces [Collins & Du Toit, 1987] (a consequence of small bowel permeability due to long-term use of NSAIDs [Bjarnason *et al.*, 1987]) would appear to indicate that there is a significant problem. The unfortunate idiosyncrasy of NSAID-induced ulcers is that they are usually 'silent' ones [Jorde & Burhol, 1987]. Whether this is a result of the drug itself masking the pain or the fact that these ulcers are in themselves asymptomatic is unknown, but what is known is that the presence of such an ulcer is usually only realised when it manifests itself on haemorrhage or perforation [Day, 1983; Langman *et al.*, 1985; Madhok *et al.*, 1986; Deakin, 1988], a catastrophic event often leading to sudden death [Armstrong & Blower, 1987; Allison *et al.*, 1992].

Enteroscopes are few (and costly) and the procedure of enteroscopy is in itself time consuming and not without risk to the patient [Morris *et al.*, 1991; 1992]. As a result there has been an increase in research into finding a means of diagnosing the presence of such ulcers, via simple diagnostic blood tests, and an attempt to learn more about the mechanisms of NSAID-induced gastroenteropathy. The six NSAID drugs selected for

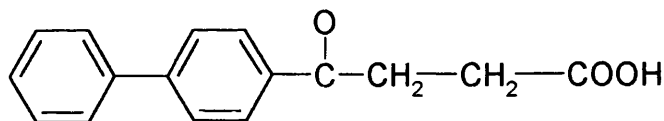
this project have been chosen because they are a cross section of such drugs on the market today. Indomethacin, which incidentally is banned in the United States of America [Spangler, 1993], is the mostly widely prescribed NSAID for rheumatoid arthritis in the United Kingdom (despite its being one of the oldest drugs of its type and being the most hazardous) as it is still the most effective in alleviating pain and inflammation. Diclofenac, which is also quite an old drug, is the most widely prescribed NSAID in Europe [Spangler, 1993] although it too is known to cause serious damage to the gastrointestinal tract (less severe than that found with indomethacin). Piroxicam and naproxen are newer than those described above (second generation NSAIDs) and as a result are less damaging to the gastrointestinal mucosa. Fenbufen and nabumetone are pro drugs (the latest generation of NSAIDs) which are inactive until they have been metabolised by the liver, thus enabling them to pass through the stomach into the small bowel without causing any damage to the gastric mucosa.

1.4.1 Diclofenac (Voltarol)



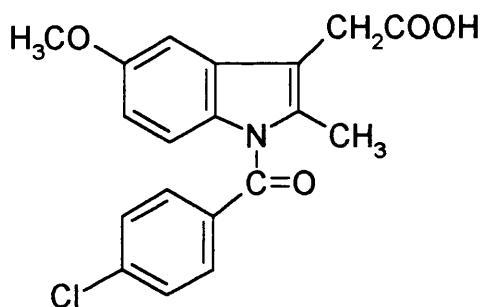
Diclofenac sodium (sodium-[*o*-[(2,6-dichlorophenyl)-amino]-phenyl]-acetate) belongs to a class of NSAIDs called Acetic Acids. Diclofenac has analgesic and antipyretic activity, is rapidly absorbed in the stomach (reaching its peak plasma concentration in man after two hours), and has a half-life of two hours. Diclofenac sodium is a weak acid with a pKa of 3.9. [Riess *et al.*, 1978; Wallis & Simkin, 1983; Brune & Lanz, 1985].

1.4.2 Fenbufen (Lederfen)



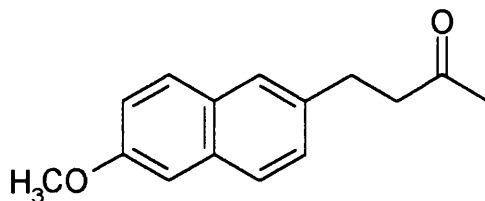
Fenbufen (γ -oxol(1,1'-biphenyl)-4 butanoic acid) is a pro-drug which belongs to a class of NSAIDs called Propionates. Fenbufen, a weak inhibitor of cyclooxygenase, is absorbed from the gastrointestinal tract and is converted by the liver into five metabolites, the most active being [1,1'-Biphenyl]-4-acetic acid (BPAA), which is a potent inhibitor of cyclooxygenase activity. Fenbufen has analgesic and antipyretic activity, is rapidly absorbed (reaching its peak plasma concentration in man after two hours (BPAA eight hours)), and has a half-life of 11 hours (BPAA 12 hours). Fenbufen is a weak acid with a pKa of 5.7. [Chiccarelli *et al.*, 1980; Birnbaum *et al.*, 1982; Brune & Lanz, 1985].

1.4.3 Indomethacin (Indocid)



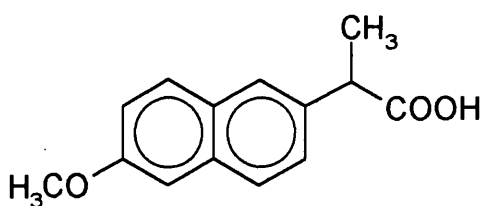
Indomethacin (1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid), an indole derivative, belongs to a class of NSAIDs called Acetic Acids. It is the most toxic of the NSAIDs, but also the most effective, and is a potent inhibitor of cyclooxygenase activity. Indomethacin has analgesic and antipyretic activity, is rapidly absorbed in the stomach (reaching its peak plasma concentration in man after two hours), and has a half-life of two hours. Indomethacin is a weak acid with a pKa of 4.5. [Yesair *et al.*, 1970; Brune & Lanz, 1985; Katzung, 1987].

1.4.4 Nabumetone



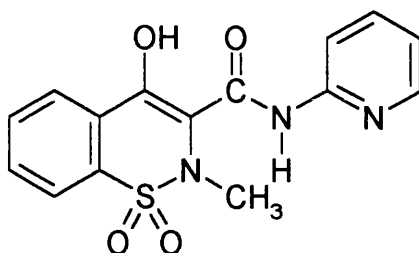
Nabumetone, a nonacidic pro-drug, belongs to a class of NSAIDs called Naphthylalkanones. Nabumetone, because it is nonacidic, cannot dissociate in the stomach and as a result 'ion-trapping' does not occur thus preventing damage to the gastric mucosa. Following absorption in the small bowel, Nabumetone, a weak inhibitor of cyclooxygenase, is converted by the liver to its active metabolite, 6-methoxy-2-naphthylacetic acid (6MNA), which is a potent inhibitor of cyclooxygenase activity. Nabumetone is well absorbed (with 6MNA reaching peak plasma concentration in man after 1 to 4 hours) and has a half-life of 24 hours. [Blower, 1992; Fleischmann, 1992; Hyneck 1992; Melarange *et al.*, 1992].

1.4.5 Naproxen (Anaprox, Naprosyn)



Naproxen [(+)-6-Methoxy- α -methyl-2-naphthalene-acetic acid] belongs to a class of NSAIDs called Propionates. Naproxen has analgesic and antipyretic activity and is rapidly absorbed in gastrointestinal tract although absorption through the stomach can be delayed if taken in conjunction with antacids. Naproxen, which is highly albumin bound, reaches its peak plasma concentration in man after two hours and has a half-life of 13 hours. Naproxen is a weak acid with a pK_a of 4.2. [Ansell *et al.*, 1975; Wallis & Simkin, 1983; Brune & Lanz, 1985].

1.4.6 Piroxicam (Feldene)



Piroxicam belongs to a class of NSAIDs called Oxicams which are members of a series of 4-(N-aryl) carboxamides of 4-hydroxy-1,2-benzothiazine 1,1-dioxide. Its chemical structure is unique as almost all other NSAIDs are basically carboxylic acids. Piroxicam has analgesic and antipyretic activity, is rapidly absorbed in the stomach (reaching 80% of its peak plasma concentration in one hour) and has a half-life of 45 hours which is long in comparison with most other NSAIDs. Unlike many NSAIDs Piroxicam is a selective reversible inhibitor of the cyclooxygenase step in the metabolism of arachidonic acid. Piroxicam is a weak acid with a pKa of 6.3. [Carty *et al.*, 1980; Brune & Lanz, 1985; Verbeeck *et al.*, 1986].

1.5 SCOPE OF THE WORK

The aim of the project was to investigate the mechanisms involved in ulceration of the stomach and small bowel caused by *Helicobacter pylori* and NSAIDs. During this research a selection of NSAIDs were used in order to compare the damage, if any, they caused to both the stomach and the small bowel. Indomethacin and diclofenac are known to cause ulceration throughout the gastrointestinal tract in man whereas nabumetone does not, thus a comparison between the different pharmacokinetic properties and actions of these drugs may possibly explain the ulcerative process and this was investigated. The rat was to be used as a model for NSAID-induced ulceration in man even though significant metabolic, inflammatory and behavioural differences were known to exist between the two species; for example drug metabolism, chemotactic factors and gut flora (due to coprophagy). Its suitability for this role was to be ascertained accordingly. In addition it was planned to investigate a potential non-

invasive diagnostic 'test' for small bowel damage by screening *in vivo* protein markers in the systemic circulation.

2. MATERIALS AND METHODS

SUPPLIERS

Agar Scientific Ltd., Stanstead, London.

Aldrich Chemical Company, Gillingham, Dorset.

All Tech Associates Inc., Applied Science Labs, Deerfield, Illinois, USA.

Amicon Ltd., Stonehouse, Gloucestershire.

Behring, Hoechst UK Ltd., Hounslow, Middlesex.

BDH, Merck Ltd. Poole, Dorset.

Bibby Sterilin Ltd., Stoke, Staffs.

Bio Mérieux, 69280 Marcy-l'Etoile, France.

Bio-Stat Diagnostics, Hazel Grove, Stockport.

BOC Gases, Bristol.

Bright Instrument Company Ltd., Huntingdon, Cambs.

Chrompack UK Ltd., Millharbour, London, E14 9TN.

Coventry Chemicals Ltd., Trowbridge, Wilts.

DAKO Ltd., High Wycombe, Bucks.

Electron Microscopy Sciences, Science Services UK Ltd., London.

Fisons (Fisher Scientific UK), Loughborough, Leics.

Flowgen, Sittingbourne, Kent.

GENLAB Ltd., Widnes, Cheshire.

Genzyme Diagnostics, West Malling, Kent.

Hayman Ltd., Witham, Essex.

HD Supplies, Aylesbury, Bucks.

HV Skan Ltd., Shirley, Solihull, West Midlands.

Interfauna, Abbots, Ripton Road, Wyton, Huntingdon, Cambs.

International Market Supply (IMS), Congleton, Cheshire.

Jackson ImmunoResearch Laboratories Inc., Stratech Scientific Ltd., Luton, Beds.

Lab M, Bury, BL9 6AU.

Leica UK Ltd., Milton Keynes

Life Sciences International (UK) Ltd., Basingstoke, Hants.

L.I.P., Shipley, West Yorks.

LKB Produkter AB, Stockholm, Sweden.

Medicell International Ltd., London, N1 1LX.
Milton Roy, Riviera Beach, Florida, USA.
Noesis, Avenue Morane Saulnier, 78140 Velizy, France.
Oxoid, Unipath Ltd., Basingstoke, Hants.
Pharmacia Biotech Ltd., St. Albans, Herts.
Pye Unicam Ltd., Cambridge, CB1 2PX.
Raymond A Lamb, London, NW10 6JC.
Scientific Lab Supplies Ltd. (SLS), Wiford, Notts.
Serotec, Kidlington, Oxon.
Sigma Chemical Company, Poole, Dorset.
Surgipath Europe Ltd., Eynesbury, St. Neots, Cambs.
Taab Laboratories Equipment Ltd., Aldermaston, Hants.
T.C.S. Biomedicals Ltd., Botolph Claydon, Bucks.
Tennants, West Bromwich, B70 0AX.
Ultratech, Kernick & Son Ltd., Cardiff.
Western Solvents Ltd., Warminster, Wilts.

2.0 MATERIALS AND METHODS - NSAIDS AND ULCER FORMATION

2.1 PRODUCTION OF SMALL BOWEL ULCERS

2.1.1 Materials

2.1.1.1 Drugs and Reagents

2.1.1.1.1 NSAIDs

Diclofenac Sodium {D-6899}, Fenbufen {F-8755}, Indomethacin {I-7378}, Piroxicam {P-5654} and Naproxen [(s)-6-methoxy-a-methyl-2-naphthaleneacetic acid free acid] {M-4015} were purchased from Sigma. Nabumetone {BRL 14777} was a gift from SmithKline Beecham Pharmaceuticals in West Sussex. Tween-80 (Polyoxyethylenesorbitan Monooleate) {P-1754} was purchased from Sigma.

2.1.1.1.2 Antibiotics

Cloxacillin Sodium Monohydrate {C-9393}, Erythromycin Ethylsuccinate {E-8755}, Nalidixic Acid Sodium {N-4382} and Neomycin Sulphate {N-1876} were purchased from Sigma. Amoxycillin and Metronidazole were purchased, on prescription from Boots the Chemists, Bath or supplied by the Pharmacy at The Royal United Hospital, Bath.

2.1.1.2 Animals

Male Wistar rats, either the University of Bath Strain or from Interfauna, maintained on a 12 hour light/12 hour dark cycle and weighing between 135 and 450 g were used in this study. Most rats weighed 250 g when dosed and calculations were made during the antibiotic studies so that when the rat was given its first dose of NSAID it weighed approximately 250 g. (One study, however, did compare the effect that size may have on the response to a particular dose of indomethacin.) Rats which were starved before dosing had food withdrawn 21 hours prior to the administration of the drug and were kept without food until 3 hours after. At all other times the rats were allowed free access to food {'Crum' from Special Diet Services (SDS)}. Water was continuously available.

2.1.2 Methods

2.1.2.1 Small Bowel Ulcers

2.1.2.1.1 Drug Preparation - NSAIDs

All the NSAIDs were dissolved/suspended in 10% Tween-80 (100% Tween-80 diluted 1:9 with distilled water). The drugs were weighed out into glass bottles, diluted to the required concentration with 10% Tween-80, vortex mixed then placed in a sonicator (Dawe 'Sonicleaner') for 30 minutes, after which time they were vortex mixed again. The drugs were dissolved/suspended in 10% Tween-80 so that the volume administered to the animal would be less than 1.0 ml. The drugs were vortex mixed again prior to administration to the animals, and between each animal dosed, if the drug was in suspension. All drugs were used within 24 hours of preparation.

2.1.2.1.2 Drug Preparation - Antibiotics

All the antibiotics were weighed into a glass bottle, dissolved/suspended in distilled water of the required volume and vortex mixed. The antibiotics were dissolved/suspended in distilled water so that the volume administered to the animal would be less than 1.0 ml. The antibiotics were vortex mixed again prior to administration to the animals, and between each animal dosed, if the drug was in suspension. All antibiotics were used within 30 min of preparation.

2.1.2.1.3 Dosing - Acute Oral Administration of NSAIDs

Groups of rats (3-6) were dosed once, orally, with one of the six drugs (diclofenac, fenbufen, indomethacin, nabumetone, naproxen, piroxicam) selected for the study and then killed 48 hours later. The doses ranged from the ID₂₅ value of each drug to five times this amount (ID₁₂₅). The ID₂₅ value being the dose calculated to bring about a 25% reduction in carrageenan-induced oedema volume in the rat [Spangler, 1993] (see table 2.1.2.1.3) which is an assay for antiinflammatory drugs [Winter *et al.*, 1962].

The ID₁₂₅ value for each drug was used initially on both starved and fed animals. In the event that ulcers were not produced, it was deemed unnecessary to dose with the ID₂₅ value, thus conserving animals. As a result, animals were only dosed with the ID₁₂₅ value

of diclofenac (11.5 mg/kg), fenbufen (50.0 mg/kg), nabumetone (79.0 mg/kg), naproxen (5.0 mg/kg) and piroxicam (7.0 mg/kg). Indomethacin ID₁₂₅ (12.5 mg/kg) caused severe ulceration so a range of doses was used in order to determine the level at which small bowel ulceration commenced and to find a dose which would reproducibly produce moderate ulceration in the small bowel. As a consequence rats were dosed with ID_{37.5}, ID₄₀, ID₅₀, ID₆₀, ID₇₀ and ID₇₅ values of indomethacin (3.75, 4.0, 5.0, 6.0, 7.0, and 7.5 mg/kg, respectively).

Compound	Oral ID ₂₅ Value mg/kg	Dose Ranges Used to Determine ID ₂₅ Value mg/kg	pKa
Diclofenac	2.3	0.3 - 10	3.9
Fenbufen	10.0	---	5.7
Indomethacin	2.5	1.0 - 10.0	4.5
Nabumetone	15.8	3.0 - 30.0	---
Naproxen	1.0	---	4.2
Piroxicam	1.4	0.3 - 3.0	6.3
ID ₂₅ is the dose calculated to produce 25% reduction in oedema volume			

Table 2.1.2.1.3 Activity of NSAIDs in Inhibiting Carrageenan-Induced Oedema in the Rat Following Oral Administration

NB. Multiples of the ID₂₅ values of the drugs used in this project do not refer to their ability to reduce carrageenan-induced oedema volume in the rat by the equivalent amount, i.e. diclofenac ID₅₀ does not reduce carrageenan-induced oedema volume by 50%. These values have been used by SKB solely as a simple means of comparing concentrations of drugs of equivalent efficacy.

In addition to the treated animals, rats were dosed with 10% Tween-80 (2.0 ml/kg) as vehicle a control and distilled water (2.0 ml/kg) as a control. Drugs were administered

to the animals using a three inch straight (16G) oral gavage needle (International Market Supply [IMS]).

2.1.2.1.4 Chronic Oral Administration of NSAIDs - Short Term

Groups of rats (3-6) were dosed once, orally, daily for seven days with one of the six drugs selected for the study, then killed 72 hours after the final dose. Initially the rats were dosed with the ID₂₅ values of the drugs diclofenac (2.3 mg/kg), fenbufen (10.0 mg/kg), indomethacin (2.5 mg/kg) nabumetone (15.8 mg/kg), naproxen (1.0 mg/kg) and piroxicam (1.4 mg/kg). The procedure was repeated using the ID₁₂₅ value of all the drugs except for indomethacin ID₁₂₅ which was omitted owing to the severity of ulceration found after a single dose. The animals dosed with diclofenac ID₁₂₅ (11.5 mg/kg), however, had to be killed on the fifth day after they had only received four doses as the rats began to lose weight. Chronic dosing was repeated using the ID₇₅, ID₁₀₀ and ID_{112.5} values of diclofenac (6.9, 9.2 and 10.35 mg/kg, respectively).

2.1.2.1.5 Chronic Oral Administration of NSAIDs - Long Term

Groups of four rats were dosed orally, once daily, for 21 days, over a four week period, with the ID₁₂₅ values of fenbufen (50.0 mg/kg), nabumetone (79.0 mg/kg), naproxen (5.0 mg/kg) and piroxicam (7.0 mg/kg). These animals were killed on the 29th day of the experiment. Owing to the severity of ulceration found after dosing with indomethacin, acutely, and diclofenac, chronically (after five days) it was decided not to use these two drugs for this particular experiment.

2.1.2.1.6 Pre-Treatment With Antibiotics - Individually

Groups of rats (3-6) were dosed orally, once daily, for three days with one of the six antibiotics selected, amoxycillin (50 mg/kg), cloxacillin (100 mg/kg), erythromycin (100 mg/kg), metronidazole (100 mg/kg), nalidixic acid (200 mg/kg) and neomycin sulphate (100 mg/kg). The rats were then given an acute dose of indomethacin 24 hours after the final dose of antibiotic and then killed 48 hours after the dose of indomethacin. (The doses administered were calculated as being equivalent to an approximation of the mg/kg value recommended for humans in the British National Formulary.)

2.1.2.1.7 Pre-Treatment With Antibiotics - Combined

Two of the antibiotics were also administered together, metronidazole (100 mg/kg) and amoxycillin (50 mg/kg) as these two antibiotics are commonly used together in clinical practice to treat various conditions affecting the human gastrointestinal tract. Groups of rats (3-6) were dosed orally, once, daily, for three days with the antibiotic mixture. The rats were then given an acute dose of indomethacin (either ID₆₀, ID₆₅ or ID₇₀) 24 hours after the final dose of the antibiotic mixture and then killed 48 hours after the dose of indomethacin. This experiment was repeated with the dose of indomethacin being administered 8 and 15 days after the three daily doses of the antibiotic mixture.

Finally a group of rats (12) were treated simultaneously. Three were dosed with the antibiotic mixture for the three days prior to 15 days before the dose of indomethacin, while the other nine were dosed with distilled water only. A week later a different group of three rats were dosed with the antibiotic mixture for three days while the remaining nine rats were given distilled water only. A week later another group of three rats were given the antibiotic mixture for three days and the remainder received distilled water only. All 12 rats were given a single dose of indomethacin (ID₆₀) 24 hours later. The animals were killed by cervical dislocation 48 hours after the dose of indomethacin.

2.1.2.1.8 Number of Animals Per Experimental Group

Owing to the variation in drug response found within Wistar rats over a prolonged period of time it was necessary to compare as many drugs as possible on animals which would react in a similar manner. In addition it was only practicable to handle nine animals at a time as on the occasions that twelve animals were killed on the same day the workload was very high and difficult to achieve within the hours that the animal house remained open. Consequently only three animals per group were used and often it was difficult to find nine animals of equivalent weight (250 g) on any one day. In the event that the results were inconclusive the experiment was repeated in order to increase the numbers within the group. On the majority of occasions the results were such that a repetition of the experiment was considered unnecessary. As a comparison between drug regimes at an instant in time these results are deemed to be valid although there are

often only three rats per group. (Similar experiments on groups equally small have been conducted in the past [Kent *et al.*, 1969].)

2.1.2.2 Sample Collection and Preparation - Blood

Blood was collected from an anaesthetised rat (Halothane) via cardiac puncture using a needle (0.8×16) and syringe (both 2.0 and 10.0 ml). For haptoglobin assays it is essential that there is no free haemoglobin in the serum. In order to achieve this the first 2.0 ml of blood (which usually enters the syringe freely under cardiac pressure) was collected separately into glass LP3 tubes {L.I.P.} using the 2.0 ml syringe as this helped to prevent haemolysis. The remaining blood was then collected into 10 ml glass Z10 tubes {L.I.P.}. The blood was left to clot for a minimum of 20 min at 4°C then spun at 2500 rpm for 15 min in a centrifuge (Jouan B311) at 4°C. Using a glass pasteur pipette the serum was aliquoted into eppendorfs (for haptoglobin or myoglobin assays) and 5.0 ml glass Z5 tubes {L.I.P.} (for stock purposes) then stored at -20°C before analysis.

2.1.2.3 Sample Collection and Preparation - Urine

Urine was collected into an eppendorf from a rat, recently killed by cervical dislocation, using a needle (0.8×16) and 1.0 ml syringe to drain the bladder. The recovery rate of urine from the animals was very poor as often the animals would urinate during the process of anaesthesia.

2.1.2.4 Removal of Small Bowel and Mapping of Ulcers.

The stomach and small bowel of each rat were removed and placed in physiological saline (0.9% NaCl (w/v) in distilled water) at 4°C and kept on ice. The stomach was opened along the greater curvature, washed with physiological saline at 4°C and inspected for signs of ulceration. The small bowel was flushed with physiological saline at 4°C using a 10 ml syringe. Once the small bowel was cleared of gut debris, it was cut into 15 cm strips, which were opened along the anti-mesenteric side and laid out onto an acetate cooled to 4°C (which was itself laid out on a glass plate cooled on the underside by ice) to expose the luminal surface. Any macroscopic lesions observed were mapped

onto a clear acetate for analysis at a later date. (The rat small bowel was usually 10 mm in width.)

2.1.2.5 Calculation of Extent of Small Bowel Ulceration

i. Densitometer

The acetates onto which the small bowel ulcers had been mapped were scanned using an Imaging Densitometer {Model GS-670, Bio-Rad}. The computer file for each individual scan was greater than the space available on a 3½ inch floppy disc (1.44 MB), so it was decided that the ulcer maps on the acetates should be reduced in size by photocopying them. However, when using the software package on the Imaging Densitometer, a discrepancy was found between the actual area of ulceration and that calculated by the computer. The problem was that the computer software analysed the density of each individual area. Consequently an area of ulceration mapped using one type of indelible marker gave the same value as a smaller area of ulceration which had been marked with a thicker pen. The lines bordering the segments were also of varying thickness which affected the overall calculation. (Even when the same pen had been used there was a difference as during the mapping process the ink lines drawn by the pen became thinner.)

ii. Graph Paper

The acetates onto which the small bowel ulcers had been mapped were photocopied. The photocopied small bowel ulcer maps were then divided into ten equal segments with a ruler and pencil. The photocopy was then overlaid with an acetate onto which a sheet of graph paper (10 mm²) had been photocopied. The area of ulceration, in square millimetres, for each section was calculated visually and then this value divided by the length of the segment in centimetres. (This converted the overall area of ulceration for each segment into a percentage figure to enable it to be compared with other animals as the lengths of the small bowels varied between animals.)

The percentage areas of ulceration were all calculated using this method since although it was time consuming it was the most accurate. The mean percentage areas were plotted

for each segment and total ulceration was calculated by summing these values and dividing by ten.

iii. Visilog

The acetates onto which the small bowel ulcers had been mapped were photocopied. The photocopied small bowel ulcer maps were divided into ten equal segments, with a ruler and pencil, which were then cut out, without the bordering lines, and stuck on a piece of A4 paper adjacent to each other,. The segments were then reduced in size, twice, using a photocopier. The reduced sections were scanned on an Imaging Densitometer and stored on a 3½ inch floppy disc (1.44 MB). The scans were then evaluated using an 'Image Processing Package' {Visilog 4, Noesis}.

The procedure of reducing the photocopies of the acetates followed by cutting and pasting ten equal segments onto another piece of paper plus the scanning process turned out to be very time consuming. In addition to this the calculation of the area of ulceration using the computer analysis software was inaccurate compared to calculating it by eye. Consequently it was decided not to use this method for calculating the percentage areas of ulceration.

2.1.2.6 Measurement of pH of Small Bowel Contents

The small bowels of control rats (250 g) were removed and laid out on a glass plate cooled on the underside by ice. Starting from the stomach end, a small cut was made every 2 cm, along the entire length of the intact small bowel, into which a piece of Universal Indicator paper (pH 1-11, Whatman) was inserted. Once this procedure had been completed the Universal Indicator papers which had been inserted first were withdrawn in chronological order. A note was made of the pH of the small bowel contents found at each point along the small bowel.

2.1.2.7 Statistical Analysis

Results are expressed as the mean (\pm SEM). The significance of the differences in the mean values within groups was assessed by Student's t-test and between groups by the

nonparametric Mann-Whitney test using the statistical software, Minitab for Windows 10.1 {Minitab Inc.}. The significance of the differences in the mean values between groups > 2 was assessed by one way analysis of variance (ANOVA) using the statistical function of Excel 5 for Windows {Microsoft Corporation}. Differences were considered statistically significant when $p < 0.05$. Regression analysis was conducted using the statistical function of Excel 5 for Windows {Microsoft Corporation}.

2.2 ISOLATION OF SMALL BOWEL MICROFLORA ASSOCIATED WITH NSAID- INDUCED LESIONS

2.2.1 Materials

2.2.1.1 Apparatus

Aluminium Test Tube Caps {TA003A, Oxoid}
Anaerobic Gas Generating Kits {BR038B, Oxoid}
Anaerobic Jar {HP011A, Oxoid}
Antimicrobial Susceptibility Discs and Disc Dispenser {Oxoid}
API 20 ϵ kit {Bio Mérieux}
AtmosBag (ethylene oxide treated) {A-3283, Sigma}
McCartney Bottles {Richardsons}
Oxy-Trap {All Tech Associates Inc.}
Pyrex Rimless Test Tubes (150 mm \times 16 mm)
Sterile Polystyrene Petri-Dishes (9 cm) {Bibby Sterilin Ltd.}
Tubing, Tygon B-44-4X {Fisons}

2.2.1.2 Culture Media

Brain Heart Infusion Medium {CM225, Oxoid}
Cooked Meat Medium {CM81, Oxoid}
Desoxycholate Citrate Agar {CM35, Oxoid}
Fastidious Anaerobic Agar {Lab90, Lab M}
Horse Blood (defibrinated) {HB034, T.C.S.}
Lab-Lemco Powder {Lab M}
McConkey 3 Agar {CM7, Oxoid}
Nutrient Agar {CM3, Oxoid}
Thioglycollate Medium {CM23, Oxoid} 1
Tryptone Soya Agar {CM131, Oxoid}
Tryptone Soya Medium {CM129, Oxoid}

2.2.2 Methods

2.2.2.1 Preparation of Growth Media

All broths and agar plates were prepared and stored as recommended by 'The Oxoid Manual' [1982].

2.2.2.2. Dissection of Small Bowel

The rat, which had been dosed once with indomethacin ID₇₅ (7.5 mg/kg), was killed (48 hours later) by cervical dislocation and the body then soaked in a mixture of 70% (v/v) ethanol/1.0% (v/v) cetrimide in distilled water to ensure that the animal was sterile on the outside. The body was then placed on a sterile dissection tray in a 'Class 3' Laminar Flow Cabinet {'Microflow' Pathfinder, Inter Med} and the small bowel dissected aseptically. (Sterile physiological saline at 37°C was poured into the peritoneal cavity so that the small bowel did not become dehydrated while it was being cut free of the mesentery, as otherwise the temperature inside the laminar flow cabinet caused the small bowel to dry out very quickly.)

2.2.2.2.1 Tissue Scrapings

The small bowel was cut into short sections (10 cm) and flushed clean of gut debris using sterile physiological saline, at 37°C, and a sterile 10 ml syringe. The section of small bowel was then opened aseptically along the anti-mesenteric side and, using a sterile (flamed) loop, scrapings were taken from the ulcer base and from the normal intact mucosa. The scrapings were placed in bottles containing either Tryptone Soya broth or Thioglycollate broth (both preheated to 37°C) and then incubated at 37°C for 48 hours. In order to obtain individual colonies aliquots of the bottle contents were then streaked out onto nutrient agar or McConkey 3 agar and incubated as before. Bacteria from individual colonies were stained using Gram's staining procedure and the gram-negative bacteria were further typed using an api 20 ϵ kit. Bacteria from distinct individual colonies, which could not be identified using the api 20 ϵ kit, were cultured in Tryptone Soya broth or Thioglycollate broth and then frozen in liquid nitrogen and stored for identification at a later date.

2.2.2.2.2 Complete Ulcers Excised Aerobically

After dissection the small bowel was supported, by laying it across sterile cocktail sticks, while short sections (10 cm) were cut off and flushed clean of gut debris using sterile physiological saline and a sterile 10 ml syringe. The sections of small bowel were then opened aseptically along the anti-mesenteric side and any ulcer found cut from the surrounding tissue. Individual ulcers, which had been excised in this manner, were cut in half and one half placed in Brain Heart Infusion broth and the other half in Cooked Meat broth (both broths preheated to 37°C) for the culture of aerobic and anaerobic bacteria, respectively. Gloves which had been tested for sterility prior to commencement of the dissection were checked similarly after the procedure had been completed. The bottles containing the broth were then incubated at 37°C and after 4 hours the broth had become turbid. At this time some of the samples were frozen in liquid nitrogen while others were streaked out onto Blood Agar, Desoxycholate Citrate Agar and Tryptone Soya Agar plates then left overnight in the incubator room at 37°C, half in anaerobic jars. The plates which had been incubated aerobically contained many colonies but the plates which had been incubated anaerobically did not, so the anaerobic jars were returned to the incubator for a further 48 hours. Bacteria from individual colonies which had been grown aerobically were stained using Gram's staining procedure and the gram-negative bacteria further analysed using an api 20 ϵ kit. As before, bacteria which could not be identified were cultured in Cooked Meat broth or Brain Heart Infusion broth and then aliquots stored in liquid nitrogen. This procedure was repeated with the slower growing bacteria which had been incubated anaerobically.

2.2.2.2.3 Complete Ulcers Excised Anaerobically

The abdomen of the rat was opened aseptically as before and the small bowel cut free of the mesentery without cutting through the small bowel itself. The small bowel was clamped at both the stomach and the caecum ends and then the small bowel was cut free of the rat on the stomach and caecum sides of the clamps, keeping the contents under anaerobic conditions. The small bowel was then immersed in sterile physiological saline, at 37°C, in a sterile plastic beaker and placed inside a sterile AtmosBag along with bottles containing either Cooked Meat broth or Brain Heart Infusion broth, which had

been preheated to 37°C. An anaerobic jar, filled with Petri dishes containing Fastidious Anaerobic Agar {Lab 90, Lab M}, was also placed inside along with small bottles of sterile physiological saline and a sterile 10 ml syringe. The bag was sealed and then filled with nitrogen. Under pressure the bag was opened slightly so that oxygenated air, which had still been trapped inside the AtmosBag, could be bled from the system. Once this was done the AtmosBag was resealed and the small bowel opened, flushed clean of gut debris and the ulcers excised as before. The ulcers were halved and one half was added to Brain Heart Infusion broth and the remaining half placed in Cooked Meat broth. Individual ulcers were also wiped across the surface of the Fastidious Anaerobic Agar. Once the lids were in place on the Petri dishes, the AtmosBag was opened and the Petri dishes placed inside an anaerobic jar. The bottles of broth and the plates in the anaerobic jar were then incubated as before at 37°C. Analysis of the cultures was performed as described above.

2.2.2.3 Preparation for Antibiotic Susceptibility Test

Samples, mixtures and isolates, which had previously been cultured from rat small bowel ulcers and stored in liquid nitrogen were grown up in Cooked Meat broth and Brain Heart Infusion broth at 37°C.

Nutrient agar plates were dried, lids off, upside down on a shelf in the incubator room for 30 min and during this time the Oxoid antibiotic disc holders were loaded into the dispensers. Once the plates were dry they were placed on the bench. A sterile Pasteur pipette was held loosely between the first two fingers while the top was taken off the bottle of medium by unscrewing the top using the small finger against the hand. Approximately 1.0 ml of medium was aliquoted onto the plate while keeping the lid of the Petri dish above it so as to prevent any airborne microbes from falling into the dish. The medium was then spread over the entire surface of the agar by tilting the dish and gently rocking. After all the dishes had been treated in this manner, the excess liquid was pipetted off the surface of the agar. Once the surface of the agar had dried the antibiotic discs were placed on the agar and the plates incubated overnight at 37°C, half aerobically and half anaerobically in anaerobic jars.

The diameter of the zones of growth inhibition were measured and a note made of any colonies of resistant populations of bacteria or of any hazy areas. The diameter of the Oxoid disk (6 mm) was subtracted from the overall diameter of circle of inhibition, and the resulting figure divided by two to give the radius of inhibition.

2.2.2.4 API 20 ϵ Kit

The api 20 ϵ kit {Bio Mérieux} is an identification system for *Enterobacteria* and other non-fastidious gram-negative rods. The system uses 23 miniaturised biochemical tests which enables 108 bacteria to be identified. The tests were carried out according to the data sheet.

2.3 ELECTRON MICROSCOPY

2.3.1 Materials

2.3.1.1 Reagents

Calcium Chloride, Magnesium Chloride, di-Sodium Hydrogen Orthophosphate Dihydrate, Sodium Dihydrogen Orthophosphate Dihydrate and Sucrose were purchased from BDH. Glutaraldehyde, Osmium Tetroxide, Paraformaldehyde and Sodium Cacodylate were purchased from Agar Scientific Ltd.

2.3.1.2 Solvents

Acetone and Ethanol were purchased from BDH.

2.3.2 Methods

2.3.2.1 Scanning Electron Microscopy (SEM)

2.3.2.1.1 Sample Collection and Preparation

2.3.2.1.1.1 Control and Experimental Rat Small Bowel Tissue

Control rats were killed by cervical dislocation. The experimental tissue included that from rats which had been dosed once, orally, either with nabumetone ID₁₂₅ (79.0 mg/kg) or indomethacin ID₇₅ (7.5 mg/kg) and from rats which had been pretreated for three days with antibiotics (metronidazole 100 mg/kg/amoxycillin 50 mg/kg) prior to the indomethacin challenge (indomethacin ID₇₅). These animals were killed 48 hours after NSAID administration by cervical dislocation. The tissue from animals with diclofenac-induced ulcers came from rats which had been dosed once, orally, for four days with diclofenac ID_{112.5} (10.35 mg/kg) which were killed 48 hours after the final dose by cervical dislocation.

2.3.2.1.1.2 Dissection of Rat Small Bowel

The small bowel was dissected out aseptically, as previously described, and cut into short sections (10 cm) then flushed clean of gut debris using sterile physiological saline, at 37°C, and a sterile 10 ml syringe. The section of small bowel was then opened aseptically along the anti-mesenteric side and discrete ulcers, surrounded by a small area

of intact mucosa, excised. These were placed in small glass jars containing 2 ml of fixative (2% (v/v) glutaraldehyde in 0.05 M Sörensen's phosphate buffer, pH 7.4) which had been pre-cooled to 4°C, capped and then stored overnight in a refrigerator at 4°C

2.3.2.1.1.3 Human Small Bowel

Samples were obtained from patients (referred by their general practitioner as presenting with upper gastrointestinal discomfort) who were undergoing upper gastrointestinal endoscopy at The Royal National Hospital for Rheumatic Diseases, Bath. The samples, taken from the upper duodenum, were placed in small glass jars containing 2 ml of fixative (2% (v/v) glutaraldehyde in 0.05 M Sörensen's phosphate buffer, pH 7.4) which had been pre-cooled to 4°C, capped and then stored overnight in a refrigerator at 4°C.

2.3.2.1.2 Scanning Electron Microscopy Processing

After overnight fixation the samples were prepared for SEM using the OTOTO method as described by Kelley *et al.* [1973] which is a modification of the OTO method first described by Seligman *et al.* [1966]. See Appendix 1.

The samples were then processed via the 'general-purpose critical-point drying method' (see Appendix 2), mounted on SEM stubs with carbon dag {Agar Scientific Ltd.} and stored with silica gel in a dessicator until required. Prior to viewing the samples were adhered to a 25 mm aluminium planchette (three samples per planchette) with carbon dag then coated with gold {Edwards 150B Gold Sputter Coater}. The samples were viewed with a JM 6310 or a T-330 Scanning Electron Microscope {Jeol}.

2.3.2.2. Transmission Electron Microscopy (TEM)

2.3.2.2.1 Sample Collection and Preparation

2.3.2.2.1.1 Control and Experimental Rat Small Bowel Tissue

Rats which had been dosed once, orally, with the ID₆₀ value of an NSAID (either diclofenac (5.52 mg/kg), indomethacin (6.0 mg/kg), nabumetone (37.92 mg/kg), naproxen (5.0 mg/kg) or piroxicam (3.36 mg/kg)) were killed 48 hours later by cervical dislocation. The control tissue came from rats which had either been dosed with distilled

water (2.0 ml/kg) or 10 per cent (v/v) Tween-80 (2.0 ml/kg) prior to cervical dislocation 48 hours.

2.3.2.2.1.2 Dissection of Rat Small Bowel

2.3.2.2.1.2.1 Method One

The small bowel was dissected out, as aseptically as possible, in a fume cupboard (using sterile apparatus and instruments) and cut into short sections (10 cm) which were then flushed clean of gut debris with fixative, at 4°C, using a sterile 10 ml syringe. The section of small bowel was then opened aseptically along the anti-mesenteric side and small pieces of tissue excised. (Samples of ulcerated and non ulcerated small bowel were taken from the animals which had been dosed with indomethacin.) Samples of all the experimental and control tissue were placed in small glass jars containing 2 ml of fixative, which had been pre-cooled to 4°C, capped and then stored on ice at 4°C.

2.3.2.2.1.2.2 Method Two

The small bowel was dissected out, as aseptically as possible, in a fume cupboard (using sterile apparatus and instruments). Small pieces (2.0 cm) containing ulcers were cut from the distal end of the small bowel and placed in a glass Petri dish containing fixative at 4°C. (The location of ulcers was determined by white nodules on the surface of the serosa.) The small bowel was cut open using a razor blade and cleaned of gut debris by gently moving it about in the fixative. Very thin sections of small bowel tissue were excised by cutting across the tissue with two razor blades, which had been taped together with a thin piece of card between them. (Samples of ulcerated and non ulcerated small bowel were taken from the animals which had been dosed with indomethacin.) Samples of all the experimental and control tissues were placed in small glass jars containing 2 ml of fixative, which had been pre-cooled to 4°C, capped and then stored on ice at 4°C.

2.3.2.2.2 Transmission Electron Microscopy - Processing

It had been reported that damaged mitochondria had been seen in the un ulcerated areas of ulcerated rat small bowel tissue taken from animals dosed with indomethacin [Bjarnason *et al.*, 1993]. As a result various combinations of recognised methods of

fixation and tissue preparation were tried, in conjunction with different resins, in an attempt to improve the visualisation of cristae within the mitochondria of rat small bowel tissue in order to try and verify this observation. On receiving advice from an expert in electron microscopy [Nunn, 1995] all samples recorded in this thesis were processed as described in Appendix 3.1. Although there was a distinct improvement in the visual quality of the sections from the blocks prepared via this particular process and it was possible to study the cristae within the mitochondria sufficiently to determine any initial damage which might be occurring, it was thought that the visual acuity could be further improved.

Finally it was decided that the tissue would have to be fixed by the perfusion method (see Appendix 3.2) and after this step the tissue was processed as for the previous samples. This time half the samples were embedded in Spurr's resin and the remainder in Quetol/NSA/MNA/ resin. During sectioning a definite improvement was found (with tissue that had been embedded in either of the two resins) as these blocks were easier to cut. The semi-thin sections, when viewed via light microscopy, were also of a higher quality than that seen previously suggesting that the tissue had been correctly fixed and that there was improved infiltration of the resin as a result. Unfortunately at this point all work in the laboratory had to cease as it was decided that insufficient time was available for any more practical work.

2.3.2.2.3 Transmission Electron Microscopy - Sectioning

2.3.2.2.3.1 Preparation of Glass Knives.

The glass knives were prepared immediately prior to use. Wearing washed gloves (powder free), a strip of ultramicrotome glass {Agar Scientific Ltd.} was first cleaned well with neat liquid detergent and a little warm water. The glass strip was then dried with a paper towel and stored in a dust free environment. The knives were cut on a LKB Knifemaker {7801A, LKB Produkter AB} during which time care was taken not to get finger prints on the glass strip. In order to cut the glass strip, it was first scored and then a straight clean fracture was obtained, along the score line, by applying equal pressure either side of it. The glass strip was first cut in half, so that it was of a more manageable size, then cut into squares. The squares were then cut into two equal halves, producing

two 45° knives, each half having one cutting edge. The knives were assessed visually for suitability and any substandard ones discarded. The selected knives were placed on a Multiplate {2208, LKB Produkter AB} to warm and then plastic troughs {LKB truf, Leica UK Ltd.} were attached to them with molten dental wax {Agar Scientific Ltd.}. The knives were stored in a glass knife storage box {Taab Laboratories Equipment Ltd.} until required for sectioning (dust particles can cause nicks in the cutting edge). At all times care was taken when handling the knives not to touch the cutting edge. (During thin sectioning a glass knife can only be used for a short period of time before it is necessary to replace it.)

2.3.2.2.3.2 Preparation and Trimming of the Block Face.

The resin block containing the sample was first pressed out of the mould or, if it had been set in a gelatin capsule, the gelatin was removed from the block with a Personna 'Super' single edged razor blade {Agar Scientific Ltd.}. The block was then inspected and mounted in the chuck in a manner which would place the sample in the best possible orientation. The block was trimmed into a trapezoidal (truncated pyramid) shape using a double-edged stainless steel teflon-coated razor blade {Electron Microscopy Sciences.} (If the sample had been embedded in LR White, a Personna 'Super' razor blade was used for trimming.) Once a flat top had been obtained the chuck was fitted into the ultramicrotome {OMU3, Reichert (Leica)}, and the top of the block fine trimmed with a glass knife.

2.3.2.2.3.3 Thin Sectioning

The block was carefully placed in the chuck (if it had been removed after trimming) and then the chuck was clamped tightly in the ultramicrotome. The knife, with plastic trough attached, was positioned in the knife holder and then moved by hand so that the block face was close to the knife but not touching.

The knife edge was aligned with the block face so that the top and bottom of the block was parallel with the knife itself. To do this the microtome lighting was switched to 'background mode' and when the knife was correctly positioned a band of bright light, seen on the block face, was of a constant width as the block passed down in front of the

knife. The knife was then carefully moved closer to the block by hand then by using the circular adjuster (which has an adjusting bolt in the top) until a thin narrow bright light with a 'rainbow effect' was seen. The plastic trough attached to the knife was filled with sterile distilled water using a 20 ml syringe which was fitted with a 0.22 μm filter. The trough was slightly overfilled at first and the excess water removed, using a needle and syringe, till an almost flat meniscus was obtained which gave a silver reflection when viewed through the binocular microscope of the microtome. (An almost flat meniscus causes optimal wetting of the knife edge.)

The knife was moved closer to the block by turning the handle and using the fine adjustment control, to the left of the microtome, until the knife started to cut sections off the block. This continued until the sections started to be cut evenly. Initially semi-thin sections of 5 μm thickness were cut which were lifted off the surface of the water with the flattened end of a cocktail stick. These semi-thin sections were then floated off onto a microscope slide which already had a pool of sterile water on it. The microscope slide was placed on a multiplate, covered by a watch glass until it dried. The semi-thin sections were then stained with 1.0 % (v/v) toluidine blue/1.0 % (v/v) borax in distilled water for one minute, rinsed with distilled water and dried again. Once dry the semi-thin sections were mounted in XAM. The semi-thin sections were then studied under a light microscope {Carl Zeiss Jena} to ensure that the sample was orientated correctly as a transverse section through the wall of the small bowel was required.

A new knife was then fitted and, once the block and the sample were correctly aligned, the ultramicrotome was switched to 'automatic mode', which meant that the knife was advanced towards the block by a thermal advance system until thin sections were cut. The thickness of these sections could be estimated by the colour they appeared to be when viewed on the surface of the water through the binocular microscope attached to the microtome (see table below). The estimated thickness of these thin sections is accurate to within 10-20 nm and the scale is based on measurements made by Peachey [1958].

Colour	Section Thickness
Grey	< 60 nm
Silver	60 - 90 nm
Gold	90 - 150 nm
Purple	150 - 190 nm
Blue	190 - 240 nm

Table 2.3.2.2.3.3 Interference colour index and section thickness scale.

Ultrathin sections of 100 nm are recommended for TEM and these appear gold on the surface of the water.) Unsuitable sections were removed from the surface of the water using an eyelash which had been attached to the end of a cocktail stick with dental wax. Ideally the gold sections formed a ribbon across the surface of the water so that as many thin sections as possible could be picked up at one time on the grid. The sections needed to be stretched prior to picking them up on the grid as they were compressed during the cutting process. This was achieved by warming them gently with a heated pen.

The sections were collected onto a metal grid (nickel, copper or gold) by holding the grid with forceps at an angle to the surface of the meniscus so that when it was removed from the water the sections were left adhering to it. The underside of the grid was dried with filter paper then left to dry completely. The thin sections were then stored in a grid box {Agar Scientific Ltd.} until required for staining and viewing. On some occasions it was necessary to use a diamond knife {DDK Delaware, Agar Scientific Ltd.} to cut thin sections because the tissue was too hard to be cut with a glass knife successfully.

2.3.2.2.4 Staining

2.3.2.2.4.1 Uranyl Acetate Solution

If the sample had not already been block stained with uranyl acetate during processing, the sections were first stained with uranyl acetate solution. The concentration, composition and length of staining time varied depending on the method of lead citrate staining which was to be used. (See Appendix 4a for details.)

2.3.2.2.4.2 Lead Citrate Solutions

A number of different lead citrate solutions were tried in an attempt to improve the degree of contrast within the ultrathin sections, when viewed via TEM.

2.3.2.2.4.2.1 Reynolds' Lead Citrate Solution (Modified)

This method of staining was first described by Reynolds [1963]. (See Appendix 4b for protocol.) However, when viewing the sections of rat small bowel on the Transmission Electron Microscope {JM 1200EX, Jeol}, the tissue was found to be inadequately stained with poor contrast.

2.3.2.2.4.2.2 Double Lead Stain

This double lead stain technique was developed by Daddow [1983] in order to enhance the contrast of tissue which had been embedded in Spurr's resin [Spurr, 1969]. (See Appendix 4c for protocol.)

Tissue may be poorly stained due to a variety of reasons one of which is that the tissue itself may be difficult to stain. Long-term fixation in glutaraldehyde and the addition of sucrose to the fixatives can also be a reason. (The osmolarity of the small bowel tissue was such that sucrose had to be added to the fixatives so that they would be of a similar osmolarity.) The double lead stain, especially the abbreviated method [Daddow, 1986], should have overcome some of the problems encountered with respect to poorly contrasting sections.

2.3.2.2.4.2.3 SynapTek Grid Stick

The SynapTek Grid Stick {Electron Microscopy Sciences} was another variation on the uranyl acetate/lead citrate method of staining sections. The staining procedure was carried out according to the data sheet.

3.0 MATERIALS AND METHODS - ROLE OF CYTOKINES

3.1 IMMUNOHISTOCHEMISTRY

3.1.1 Materials

3.1.1.1 Apparatus

Cryostat Tubes {Corning, BDH}

Dialysis Tubing (Size 1) {Medicell International Ltd.}

Glass Racks {E/98, R.A. Lamb}

Glass Troughs {E/105 & E/106, R.A. Lamb}

Incubator/Steriliser (MINO/30/SS/IS) {SS20, GENLAB}

'Pen for Immunocytochemistry' {S2002, DAKO}

Plastic Slide Box { E32.6, R.A. Lamb}

Polyacetal Rack {E99, R.A. Lamb}

Polypropylene Graduated Disposable Pasteur Pipettes (1 and 3 ml) {S.L.S.}

Polypropylene LP3 Tubes {30882, L.I.P.}

Polypropylene Caps for LP3 Tubes {30965, L.I.P.}

Polystyrene LP3 Tubes {30908, L.I.P.}

Polystyrene Z5 Tubes {71050 Z5/WSC, L.I.P.}

Prestige Stainless Steel Automatic Pressure Cooker {860/7223, Argos}

Pre-Washed Microscope Slides (Twin Frosted One End) {HV Skan Ltd.}

Stainless Steel Swing Handle Rack {E/102, R.A. Lamb}

Wire Lifters for Glass Racks {E/97.5, R.A. Lamb}

Work Station {DAKO}

3.1.1.2 Reagents

ABComplex/HRP {K0355, DAKO}

Aluminium Potassium Sulphate 12-Hydrate {10009}, Ammonium Sulphate {10033}, Calcium Chloride 2-hydrate {10070}, Chloral Hydrate {27668}, Haematoxylin (monohydrate) {34037}, Hydrogen Peroxide {10128}, Tris[hydroxymethyl]methylamine {10315} and XAM neutral medium improved white Gurr {36119} from BDH.

Hydrochloric Acid (S.G.1.18), Potassium Dihydrogen Orthophosphate, di-Sodium Hydrogen Orthophosphate, Sodium Chloride and Sodium Hydroxide from Fisons.

Gill2 Hematoxylin {Shandon, Life Sciences International}

3-Aminopropyltriethoxysilane (APES) {A-3648}, Bacterial Lipopolysaccharide (LPS) {L-8274}, Bovine Serum Albumin {A-7284}, A-Chymotrypsin Type II From Bovine Pancreas {C-4129}, 3,3'-Diaminobenzidine (DAB) Tetrahydrochloride Tablets {D-5905}, Poly-L-Lysine {P-8920}, Sodium Azide {S-2002}, Sodium Citrate Dihydrate ACS Reagent {S-46410} from Sigma, Sodium Iodate {21,591-0, Aldrich}.

RPMI 1640 Medium and Foetal Calf Serum from GIBCO.

Eosin Yellow {S/115.3} and OCT (Tissue Tek){C/101.25} from R.A. Lamb

Sodium Hyperchlorite {Coventry Chemicals Ltd.}

Sta-On {Surgipath}

3.1.1.3 Solvents

Buffered Formalin Concentrate {Western Solvents Ltd.}

Citroclear CC500 {HD Supplies}

Ethanol {Hayman Ltd.}

Industrial Methylated Spirits 99 {Tennants}

Isopentane (2-Methylbutane) {10361, BDH}

Acetone and Methanol (both HPLC Grade) {Fisons}

Xylene {BDH}

3.1.1.4 Primary Antibodies - Rat Studies

Polyclonal Rabbit Anti-Mouse IL- α {IP-110, Genzyme}

Monoclonal Hamster Anti-Mouse IL- β {1997-01, Genzyme}

Monoclonal Mouse Anti-Human IL-4 {1842-01, Genzyme}

Polyclonal Rabbit Anti-Mouse TNF- α {IP-400, Genzyme}

Polyclonal Rabbit Anti-Mouse TNF- α {Gift from Dr. S.L. Kunkel, University of Michigan, USA.}

3.1.1.5 Primary Antibodies - Human Studies

Polyclonal Rabbit Anti-Human IL-1 α {80-3054-01, Genzyme}

Monoclonal Mouse Anti-Human IL-8 {MCA1109, Serotec}

Polyclonal Rabbit Anti-Human IL-8 {80-3718-01, Genzyme}

Monoclonal Mouse Anti-Human IL-8 {Gift from Dr. Ivan Lindley, SANDOZ, Vienna, Austria.}

Polyclonal Rabbit Anti-Human TNF- α {IP-300, Genzyme}

3.1.1.6 Secondary Antibodies

Biotinylated Swine Anti-Rabbit Immunoglobulins {E353, DAKO}

Biotinylated F(ab')₂ Fragment of Rabbit Anti-Mouse {E413, DAKO}

Biotinylated Rabbit F(ab')₂ Fragment Rabbit Anti-Syrian Hamster {307-066-003} and

Biotinylated Rabbit Anti-Syrian Hamster IgG {307-065-003} from Jackson ImmunoResearch.

3.1.1.7 Recombinant Proteins - Rat Studies

Recombinant Mouse IL-1 α {1920-01, Genzyme}

Recombinant Mouse TNF- α {TNF-M, Genzyme}

Rat Interleukin-4 {Gift from Serotec}

3.1.1.8 Recombinant Proteins - Human Studies

Recombinant Human IL-1 α {Gift from Glaxo}

Recombinant Human TNF- α {Gift from Bayer}

3.1.1.9 Control Serums

Normal Swine Serum {X0901}, Normal Rabbit Serum{X0902} and Normal Rabbit Immunoglobulin Fraction {X0903} were purchased from DAKO.

Normal Rabbit Serum {R-4505, Sigma}

3.2 Methods

3.2.1 Sample Collection and Preparation.

3.2.1.1 Positive Control Tissues

Positive control tissues were collected so that they could be used during the immunohistochemistry procedure. The specimens are processed identically to the experimental tissue but must contain the target protein. It is advantageous, although not necessary, to have the control tissue stain marginally positive so as to monitor not only for the presence of the antigen but also any possible loss of sensitivity. This loss might not be apparent if only intensely staining controls are used [Boenisch 1989].

3.2.1.1.1 Rat Lung

Rat lung was incubated with bacterial lipopolysaccharide (LPS), 10 µg/ml, in RPMI 1640 medium containing 10% foetal calf serum for 1, 2 and 4 hours at 37°C. After incubation the tissue was either placed in 10% neutral phosphate buffered formalin or snap frozen. Sections taken from the formalin-fixed paraffin-embedded activated lung tissue, which had been incubated for one hour with LPS, were found to have sufficient TNF-α and IL-1α activity for binding of the rabbit anti-mouse TNF-α and the rabbit anti-mouse IL-1α so this was the control tissue used for these two antibodies.

3.2.1.1.2 Rat Spleen

Initially a rat spleen was dissected out of the animal, placed in 10% neutral buffered saline and then embedded in paraffin wax. However, when sections of this tissue were used for immunohistochemistry the activity of TNF-α was so low that no visible binding of the polyclonal rabbit anti-mouse TNF-α was detected. Therefore it was decided to activate the spleen with bacterial lipopolysaccharide (LPS) [Zuckerman *et al.*, 1991]. Initially rat spleen was incubated with LPS, 10 µg/ml, in RPMI 1640 medium containing 10% foetal calf serum for 1, 2 and 4 hours at 37°C. After incubation the tissue was either placed in 10% neutral phosphate buffered formalin or snap frozen in isopentane/liquid nitrogen. Although sections from the formalin-fixed paraffin-embedded tissue displayed positive binding for the rabbit anti-mouse TNF-α and the rabbit anti-

mouse IL-1 α it was not as suitable as the lung tissue. In the literature spleen was reported as being an organ which could be induced to synthesise an abundance of IL-1 β [Koenig *et al.*, 1990], however no such activity was found initially. Further research of the literature revealed that levels of IL-1 β did not start to rise until six hours after initial stimulation with LPS and reached peak levels after approximately 19 hours [Lonnemann *et al.*, 1989]. As a result rat spleen was incubated as before but this time for six hours and 19 hours.

3.2.1.1.3 Human Tonsil

Human tonsil tissue was collected, within minutes of its removal from the patient, from the operating theatres of the Royal United Hospital, Bath. The tissue was rapidly cut into small segments which were either fixed in 10% neutral phosphate buffered formalin or snap frozen in isopentane/liquid nitrogen. At the time a note was made as to the suitability of the tissue collected for control tissue purposes as the tonsil tissue needed to be 'reactive' but not too infected. Tonsil tissue was selected for this purpose as it has been shown that tonsil tissue produces 19 different cytokines [Andersson *et al.*, 1994].

3.2.1.2 Experimental Tissues

3.2.1.2.1 Rat Small Bowel

Groups of rats (3-6) were dosed once with either indomethacin, ID₆₀ (ulcer causing dose) or ID₃₀ (non ulcer causing dose), 10% Tween-80 (vehicle) or with water (control). 48 hours later they were killed by cervical dislocation and samples were taken for immunohistochemistry.

3.2.1.2.2 Human Gastric Mucosa

Samples of gastric mucosa (5 per patient) were obtained from patients (referred by their general practitioner as presenting with upper abdominal discomfort) who were undergoing upper gastrointestinal endoscopy at The Royal National Hospital for Rheumatic Diseases, Bath. Immediately after excision two samples were placed in plastic universals containing 10% neutral phosphate buffered formalin and two were snap frozen in isopentane/liquid nitrogen. The fifth sample was placed in the medium of a

'CLO Test' (for *Campylobacter*-like organisms), containing urea and a pH-sensitive dye [Marshall *et al.*, 1987], to see if the sample was positive for *Helicobacter pylori*. At endoscopy a note was made as whether the patient was *Helicobacter pylori*-positive and whether or not they had ulcers or were suffering from gastritis.

3.2.1.3 Formalin-Fixed Paraffin-Embedded Tissue

3.2.1.3.1 Histopathology Laboratory, Royal United Hospital, Bath.

Samples were placed in plastic containers (universals or Sterilin 50 ml pots) containing 10% neutral phosphate buffered formalin. (It is imperative for immunohistochemistry that the samples be embedded as soon as possible, recommended fixation time being between 6-24 hours.) After fixation the samples were processed overnight on an enclosed processor {Shandon Hypercenter 2} and embedded in paraffin wax {Histoplast, Life Sciences International} which is a blend of paraffin waxes that has a melting point at 56°C (see table 3.2.1.3.1).

Programme For Shandon Hypercenter 2					
PROGRAMME		ROUTINE			
	Reagent. %	TMP	VAC	TIME	DRAIN
1.	Formalin 10%	40	N	2.15	1
2.	Alcohol 70%	37	N	0.25	1
3.	Alcohol 95%	A	N	0.25	1
4.	Alcohol 100%	A	N	0.25	1
5.	Alcohol 100%	A	N	1.00	1
6.	Alcohol 100%	A	N	1.00	1
7.	Alcohol 100%	A	N	1.00	1
8.	Xylene	A	N	1.00	1
9.	Xylene	A	N	1.00	1
10.	Xylene	A	N	1.30	1
11.	Wax	62	Y	1.30	2
12.	Wax	62	Y	2.30	2
Total Time		14 Hrs 36 Mins			

Table 3.2.1.3.1 Processing Procedure for Formalin-Fixed Paraffin Embedded Tissue

3.2.1.3.2 Bath Clinic, Bath.

Samples were placed in plastic universals containing 10% neutral phosphate buffered formalin. After fixation the samples were processed overnight and embedded in plasticised paraffin wax {Life Sciences International} at a temperature of 65°C.

3.2.1.4 Samples frozen in isopentane/liquid nitrogen

3.2.1.4.1 Rat Lung, Rat Spleen, Human Gastric Mucosa and Human Tonsil

A 50 ml plastic beaker containing approximately 30 ml of isopentane was lowered into a thermos flask, using a wooden test tube holder, containing liquid nitrogen. Once the isopentane had frozen solid it was removed from the liquid nitrogen and allowed to partially thaw. The samples were dropped into the isopentane and once frozen were placed into cryostat tubes and then dropped directly into the liquid nitrogen. The samples were then stored in a freezer at -70°C. Some samples were surrounded by OCT (Tissue Tek) and wrapped in aluminium foil before being dropped into the isopentane and then these packets were dropped into the liquid nitrogen. These samples were then placed into airtight polythene bags and also stored at -70°C.

3.2.1.4.2 Rat Small Bowel

Samples of rat small bowel were cut down the antimesenteric side and laid out carefully onto benchcoat. The small bowel samples and the benchcoat were then dropped into isopentane at the correct temperature. These samples, which had been frozen flat, were carefully removed from the benchcoat and then put into cryostat tubes and dropped into liquid nitrogen. (After being frozen in the isopentane an attempt was made to freeze some of these frozen samples in OCT (Tissue Tek) but the problem was that the small bowel samples defrosted too quickly and could not be frozen flat using the OCT.) The samples were then stored in a freezer at -70°C.

3.2.2 Preparation of Glass Slides for Sections Cut on Cryostat

Glass slides were first placed in a plastic slide rack and then cleaned in acid alcohol (1% (v/v) concentrated HCl in 70% (v/v) ethanol) for 10 min. The glass slides were then washed well in distilled water.

3.2.2.1 Poly-L-Lysine

The poly-L-lysine (1 g/l) was diluted 1:10 with deionized water prior to coating the glass slides. Cleaned glass slides in a plastic rack were placed in the poly-L-lysine solution, held in a 1 litre polypropylene beaker, for 5 min. The slides were then drained and either dried for one hour in an oven at 60°C or at room temperature overnight.

3.2.2.2 'Sta-On'

A 5% solution (50 ml/l) of 'Sta-On' {Surgipath} was used for precoating the glass slides. Cleaned glass slides in a plastic rack were placed in the 'Sta-On' solution for 30 minutes then drained and left to dry at room temperature.

3.2.3 Sectioning

3.2.3.1 Formalin-Fixed Paraffin-Embedded Tissue

3.2.3.1.1 Histopathology Laboratory, Royal United Hospital, Bath.

The sections were cut, at a thickness of 4 μ m, on a Leitz Rotary Microtome and floated off into a water bath, at 43°C, containing a tissue section adhesive {'Sta-On', Surgipath, 10 ml/l}. (Initially glass slides pre-coated with 2% (v/v) APES were used but owing to a problem with the sections washing off the slides during the immunohistochemical procedure it was decided to switch to 'Sta-On'). The sections were then picked up on glass slides, which had been acid/alcohol washed, and dried for between 24 and 48 hours in an oven at 37°C. A section from each block was H & E stained to ensure that the tissue was correctly orientated and that the sections were of a high enough quality for immunohistochemistry

3.2.3.1.2 Bath Clinic, Bath

Initially the sections were cut, at a thickness of 4 μ m, on a Leitz Sledge Microtome and floated off into a water bath at 55°C containing 'Sta-On', 10 ml/l, a tissue section adhesive. The sections were then picked up on glass slides, which had been acid/alcohol washed, and dried for 1 hour at 70°C. This method of preparing tissue sections is suitable for routine light microscopy but not for sections which are required for

immunohistochemistry. Once this mistake had been realised the sections were cut and dried at temperatures as close as possible to those recommended by the Royal United Hospital.

3.2.3.2 Frozen Tissue

3.2.3.2.1 Cryostat, School of Pharmacy and Pharmacology, University of Bath.

Some OCT (Tissue Tek) was placed on the cooled chuck in the cryostat and allowed to freeze. The tissue was then placed on this frozen OCT, surrounded by more OCT, orientated into the correct position and held in place until the OCT froze around it. The sections were cut, at a thickness of 6 μm , on a cryostat {Bright Instrument Company Ltd.} at a temperature of -12 to -15°C and picked up onto precoated glass slides which were themselves at room temperature.

Problems were encountered when trying to cut sections of 6 μm thickness on this cryostat as the knife was not sharp enough to cut the tissue cleanly and the sections were torn. An attempt was made to use these sections for immunohistochemical staining but the antibodies became trapped under the sections where the tissue was ripped.

There was also an additional problem with cutting human tonsil tissue on this cryostat as it was in regular use by other members of the department who had not been vaccinated against Hepatitis B. Therefore it was decided to seek permission to cut the sections on the cryostat in the Histopathology Laboratory at the RUH.

3.2.3.2.2 Cryostat, Histopathology Laboratory, RUH, Bath.

Some OCT (Tissue Tek) was placed on the cooled chuck in the cryostat and allowed to freeze. The tissue was then placed on this frozen OCT, surrounded by more OCT, orientated into the correct position and held in place until the OCT froze around it.

The sections were cut, at a thickness of 5 μm , on a cryostat {1720, Leitz} at a temperature of -20°C and picked up onto precoated glass slides which were themselves at room temperature (3 sections/slide). The first few sections from each sample were H

& E stained to ensure that the tissue was correctly orientated and that the sections were of a high enough quality for immunohistochemistry. During sectioning the slides were stored in a rack inside the cryostat until sufficient sections had been cut. The sections were then air dried (by placing the slide rack in front of a fume cupboard which had the front closed down) for 30 minutes at room temperature. The sections were then stored at -70°C in one of three ways (detailed in chronological order).

(i) The sections were placed in a large plastic slide holder with some silica gel, wrapped in aluminium foil and sealed with masking tape.

(ii) The sections were placed in small plastic slide holders, (5 sections/holder), sealed with masking tape and then placed into airtight polythene bags with silica gel.

(iii) The sections were placed in cardboard slide holders, wrapped well in aluminium foil, sealed with masking tape and then placed into airtight polythene bags with silica gel.

The final method of storage was found to be the most suitable and only enough slides for an experiment were sealed up in each individual packet.

3.2.4 Antibodies

3.2.4.1 Commercially Available Antibodies

The antibodies which had been purchased were stored on arrival at the temperature recommended by the data sheet. Prior to use 10 µl aliquots were pipetted aseptically into sterile 0.5 ml eppendorfs which were then frozen upright in a plastic rack. (The antibodies were aliquoted to prevent a freeze/thaw cycle which would destroy the antibody.) The frozen aliquots were placed in suitable containers which were then sealed inside airtight polythene bags in order to prevent the small aliquots from dehydrating whilst stored in the freezer. Most of the antibodies were stored at -20°C however the monoclonal hamster anti-mouse IL-1 β was stored at -70°C as recommended. The biotinylated F(ab')₂ fragment rabbit anti-syrian hamster IgG secondary antibody had to be mixed 1:1 with sterile glycerol before it was stored in the freezer at -20°C.

3.2.4.2 Donated Antibodies

3.2.4.2.1 Polyclonal Rabbit Anti-Mouse TNF- α

3.2.4.2.1.1 Hyperimmune Serum

This antibody, which was a gift from Dr Kunkel, was stored as a hyperimmune serum in aliquots of varying sizes. Prior to use, for immunohistochemistry, 10 μ l aliquots were pipetted aseptically into sterile 0.5 ml eppendorfs which were then frozen upright in a plastic rack. (The antibodies were aliquoted to prevent a freeze/thaw cycle which would destroy the antibody.) The frozen aliquots were placed in suitable containers which were then sealed inside airtight polythene bags in order to prevent the small aliquots from dehydrating whilst stored in the freezer.

3.2.4.2.1.2 Rabbit Gamma-Globulin (R γ G) Preparation

The polyclonal rabbit anti-mouse TNF- α had been supplied as a neat hyperimmune serum. In order to improve the binding of this antibody it was decided that it should be further purified. The method of ammonium sulphate precipitation used was a modification of that described by Hudson & Hay [1989]. (The ammonium sulphate precipitate of whole rat serum is not strictly γ -globulin but is serum depleted mainly of albumin.)

3.2.4.2.1.2.1 Antiserum Fractionation

Hyperimmune rabbit serum (1.5 ml) was placed, with a small flea, in a Beckman centrifuge tube, which was held over a magnetic stirrer by a clamp fixed to a retort stand. To this 1.5 ml of saturated ammonium sulphate solution (in 0.2 M Tris, pH 8.0) was added dropwise, with constant stirring, to 50% saturation. The resultant mixture was left stirring at room temperature for 30 minutes. The mixture was then spun at 6000 rpm in a Beckman J2-MC centrifuge, using the 20.1 rotor, for 20 min at 25°C. (The outer holes of the rotor were at a radius of 115 cm which meant that the mixture was spun at approximately 3000g as required.) The supernatant was pipetted off and stored at 4°C and the precipitate was redissolved in 1.5 ml of 0.2 M Tris-HCl, pH 8.0. To this 1.5 ml of saturated ammonium sulphate solution (in 0.2 M Tris, pH 8.0) was added dropwise, with constant stirring, to 50% saturation. The resultant mixture was left stirring at room

temperature for 30 minutes and then spun as before. The supernatant was pipetted off and stored at 4°C and the precipitate was redissolved in 1.5 ml of 0.02 M phosphate buffer, pH 7.4. The solution was then loaded into dialysis tubing and the Beckman tube rinsed with a small amount of phosphate buffer which was also loaded into the dialysis tubing. The tubing was knotted and the solution inside dialysed at 4°C against 2.0 litres of phosphate buffer pH 7.4., twice for 2 hrs, overnight and then 4 hours in phosphate buffer which had been made up with Milli-Q water.

A precipitate had formed during dialysis so the RyG was spun as before. The supernatant was then diluted 1:19 with phosphate buffer and read at 280 nm in a 0.8 ml quartz cuvette on a spectrophotometer (UV/VIS, Lambda 3, Perkin-Elmer). (At 280 nm an Optical Density of 1.0 (in a 1.0 cm cuvette) \equiv RyG conc of 0.74 mg/ml.) The two original supernatants were discarded when it was known that a good yield of RyG had been obtained. In the event that the concentration of protein had been very low, the two original supernatants would have been combined and the whole process repeated using the combined supernatants.

This procedure was repeated at a later date with 250 μ l of hyperimmune serum and although the yield was slightly down (more dilute due to the smaller quantity being used) it was very successful. Initially the RyG of the hyperimmune serum was stored in 50 μ l aliquots at -20°C however, after 6 months, the antibody was found to have lost its activity. As a result it was decided to store subsequent batches of RyG at 4°C with 0.1% (w/v) sodium azide.

3.2.4.2.1.2.2. Control Rat Serum Fractionation

The above purification method was repeated using 1.5 ml of frozen serum from a Male Wistar Rat, University of Bath Strain.

3.2.4.2.2 Monoclonal Mouse Anti-Human IL-8

This was stored, as delivered, with 0.1% (w/v) sodium azide at 4°C.

3.2.5 Preparation of Dialysis Tubing

Approximately 30 cm of dialysis tubing was placed in a glass beaker with 500 ml of Milli-Q water (containing a few mg of EDTA) and boiled for 10 min. The dialysis tubing was then washed well with Milli-Q water, and stored at 4°C in Milli-Q water with sodium azide (0.05% w/v) until required for use.

3.2.6 Haematoxylin and Eosin Staining

See Appendix 5

3.2.7 Procedures for Immunohistochemistry

The technique chosen for the immunohistochemistry procedure was that which uses the Avidin-Biotin-Peroxidase Complex (ABC) which was developed by Hsu *et al.* [1981] for use with formalin-fixed paraffin-embedded tissue. The binding of this complex to the biotinylated secondary antibody results in a higher staining intensity, owing to amplification of the original signal, as a large amount of peroxidase is attached to each primary antigen site. The peroxidase is then developed by DAB (3,3'-Diaminobenzidine Tetrahydrochloride). This peroxidase staining method was selected, rather than one which utilised alkaline phosphatase, because it enabled the stained sections to be stored over a long period of time without fear of the staining in the tissue fading. (Staining with alkaline phosphatase only remains visible for a few weeks.) The problem with using a peroxidase staining method is that the tissue sections have to be blocked with 2.5% (v/v) Hydrogen Peroxide/Methanol, to quench any endogenous peroxidase activity, and there is a risk that some antigens may be destroyed by this procedure.

3.2.7.1 Formalin Fixed Paraffin Embedded Tissue

3.2.7.1.1 Monoclonal Antibodies

Protocol - See Appendix 6

During the immunohistochemical procedure negative control sections are processed at the same time. These sections of positive control tissue and experimental tissue are subject to the same procedure as the other sections except that they are incubated with buffer

only when the other sections are being incubated with the primary antibody. These control sections are to ensure that there is no non-specific staining present and should always be free of staining after the procedure is completed. Should any staining be found in these sections it may be due to either (or all) of the following.

In order to find out which of the steps in the process is responsible it is necessary to run the immunohistochemical procedure, using sections of positive control tissue without using a primary antibody and omitting each one of the subsequent staining steps in turn, replacing it with buffer.

i. Endogenous Peroxidase Activity

Staining obtained when both the secondary antibody and the ABComplex have been replaced with buffer means that the DAB is binding to the endogenous peroxidases in the tissue and the Hydrogen Peroxidase/Methanol blocking step needs to be extended in time or the concentration of hydrogen peroxidase increased.

ii. Endogenous Biotin

Staining obtained when the secondary antibody has been replaced with buffer is indicative of the ABComplex binding to endogenous biotin in the tissue.

iii. Non-Specific Binding of Secondary Antibody

Should the above both give a negative result then the non-specific staining must be due to the secondary antibody. This can be overcome by using the F(ab')₂ fragment of the biotinylated antibody instead.

3.2.7.1.2 Polyclonal Antibodies

Protocol - See Appendix 7

During the immunohistochemical procedure negative control sections are processed at the same time as described above. In addition to these, sections of positive control tissue and experimental tissue are processed using control serum (from the same animal that the

primary antibody had been raised in), at the same concentration but instead of the primary antiserum. (When using the IgG fraction of the antiserum the equivalent fraction is used from the control serum.) This is to ensure that all visible staining is due to the antibody binding to the protein under investigation only.

3.2.7.1.3 Antigen Retrieval

3.2.7.1.3.1 Protease (Trypsin)

Distilled water (300 ml) and tris buffered HCl, pH 7.8, (300 ml) were placed in glass pots then warmed in an oven at 37°C. Once warm the glass pot containing tris buffered HCl was placed on a magnetic stirrer (heater on very low) with a flea. To this calcium chloride (0.3 mg) and α -chymotrypsin (0.3 mg) were added. Once these reagents had dissolved sodium hydroxide (3%) was added until the solution was at a pH of 7.8. The trypsin solution was then returned to the oven until required for use.

After deparaffinisation and blocking with methanol the sections were washed and then placed in warm distilled water for 5 minutes at 37°C. Next the sections were placed in the trypsin solution and returned to the oven for 15 minutes at 37°C. The sections were then washed well with water and placed in a glass pot containing tris buffered saline, pH 7.6, followed by blocking with serum which had come from the animal in which the secondary antibody had been raised. The protocol for immunohistochemistry then proceeded as before.

3.2.7.1.3.2. Microwaving

The technique used was based on that described by King [1994]. After deparaffinisation and blocking with methanol the sections, in a polyacetyl rack, were washed well and then placed in a polypropylene container (measuring 140 mm × 90 mm × 80 mm) with 600 ml of 0.01M HCl sodium citrate buffer, pH 6.0. The height of the liquid in the container was marked, with an indelible pen, and then the container was placed into a microwave oven (Blomberg 850 w). The container and sections were heated on full power for a total of 40 minutes in 4 × 10 minute bursts. After each 10 minute period the level of liquid in the container was topped up with distilled water to the level marked initially.

(The container with the sections was itself placed in a larger polypropylene box so that if the buffer boiled over it would be contained.) The sections were then left for 20 minutes to cool to room temperature and then transferred into a glass pot containing tris buffered saline, pH 7.6. The protocol for immunohistochemistry then proceeded as before with the serum blocking step.

3.2.7.1.3.3. Pressure Cooking

The technique used was based on that described by Auld [1994]. After deparaffinisation and blocking with methanol the sections, in a stainless steel rack, were washed well with water. During these procedures 3.0 litres of 0.01M HCl sodium citrate buffer, pH 6.0, were brought to the boil in a stainless steel pressure cooker, BUT NOT under pressure. The sections were carefully lowered into the boiling buffer, the pressure cooker lid closed, and then, after full pressure had been obtained, boiled for 2 minutes. The pressure cooker was cooled rapidly by quenching in a sink of cold running tap water. Once cooled the sections were washed well in water and then transferred to a glass pot containing tris buffered saline, pH 7.6. The immunohistochemistry protocol was then followed as before.

N.B. Stainless steel apparatus had to be used during pressure cooking so that the buffer was not contaminated with aluminium ions.

3.2.7.2 Frozen Sections - Protocol

3.2.7.2.1 Monoclonal and Polyclonal Antibodies

Sections were removed from the freezer and left to come to room temperature, still wrapped up, for 30 minutes to 1 hour. The sections were fixed in acetone at room temperature for 10 minutes and then left to air dry for 10 minutes. The sections were then blocked in 2.5% (v/v) hydrogen peroxide in methanol for 15 minutes followed by rinsing in distilled water. The sections were placed in a glass pot of tris buffered saline, pH 7.6 for 5 minutes and then the procedure for immunohistochemistry proceeded as normal with the serum blocking step.

Initially the sections were blocked in 0.6% (v/v) hydrogen peroxide in methanol for 20 minutes, as recommended by DAKO, however background staining was found in the negative control section due to the DAB binding to the endogenous peroxidase in the tissue. An experiment without using a primary antibody was conducted to see what the minimum length of time was required for blocking. It was found that for most frozen tissue blocking in 2.5% (v/v) hydrogen peroxide in methanol for 15 minutes was sufficient.

3.2.7.2.2 Antigen Retrieval - Saponin

The method used was that described by Andersson *et al.* [1994].

3.2.8 Protein Blotting

The protein blotting technique used was an adaptation of two methods previously described by Renart *et al.* [1979] and Bowen *et al.* [1979]. (See Appendix 8 for protocol.)

3.2.9 Neutralisation of Mouse IL-1 α and Mouse TNF- α

The neutralisation procedure was necessary in the case of the polyclonal rabbit anti-mouse IL-1 α and the polyclonal rabbit anti-mouse TNF- α as these antisera were not specific for the respective rat cytokines. Although the Genzyme polyclonal rabbit anti-mouse IL-1 α had been reported as crossreacting with rat IL-1 α [Diamond & Pesek, 1991; Perretti *et al.*, 1991, Merrick *et al.*, 1992, Merrill *et al.*, 1993; Teti *et al.*, 1993] as had the Genzyme polyclonal rabbit anti-mouse TNF- α [Diamond & Pesek, 1991; Merrill *et al.*, 1993; Teti *et al.*, 1993] and the donated polyclonal rabbit anti-mouse TNF- α [Osborn *et al.*, 1989] it was still necessary to confirm this with the sections of rat tissue being used for immunohistochemistry.

3.2.9.1 LP3 Tubes

Recombinant protein, of varying concentration, in 100 μ l of tris buffered saline (pH 7.6), was added to LP3 tubes with 100 μ l of double strength working antibody dilution. The solutions were mixed then left to stand for one hour. The mixtures were then added to

sections of control tissue, at the primary antibody incubation step, and the protocol for immunohistochemistry followed as before.

3.2.9.2 ELISA Plate

Recombinant protein, of varying concentration, in 100 μ l of coating buffer (0.05 M sodium carbonate buffer, pH 9.6), was added to a 96-well ELISA plate, sealed with a transparent lid and left overnight at 4°C (or 2 hours at 37°C). The following morning the coating buffer was aspirated from the wells and the wells were rinsed twice with tris buffered saline (pH 7.6). A 200 μ l aliquot of tris buffered saline (pH 7.6) containing the antiserum (working dilution) was added to each of the wells and left for 1 hour at 37°C. The contents of each well were then added to sections of control tissue (one well per section), at the primary antibody incubation step, and the protocol for immunohistochemistry followed as before.

4.0 MATERIALS AND METHODS - *IN VIVO* PROTEIN MARKERS OF SMALL BOWEL LESIONS

4.1 HAPTOGLOBIN

4.1.1 Materials

4.1.1.1 Apparatus

4.1.1.1.1 Radial Immunodiffusion (RID)

Haptoglobin Kit RID Method { Hycor Biomedical Inc., 883145, Bio-Stat }

4.1.1.1.2 Immuno-electrophoresis

Agarose 15 'Electran' {44302, BDH}

Gel Bond-Agarose Support Medium (102 mm × 0.2 mm × 16.5 m) {Flowgen}

Gel Levelling Platform

Glass Gel Support Plates

Multiphor II Electrophoresis System {Pharmacia}

Spirit Level

Whatman Filter Paper

4.1.1.2 Reagents

Barbitone (Diethylbarbituric Acid) {27282}, Barbitone Sodium {27283} and

Polyethylene Glycol (PEG) 6000 {44271} were purchased from BDH. Brilliant Blue R (Coomassie Brilliant Blue R-250) {B-0149, Sigma}

4.1.1.3 Solvents

Glacial Acetic Acid {A/0360/PB17, Fisons} and Methanol {BDH}

4.1.1.4 Antibodies

Polyclonal Goat Anti-Human Haptoglobin {ORER 04/05, Behring}

Polyclonal Rabbit Anti-Human Haptoglobin {A0030, DAKO}

4.1.1.5 Standard and Control Serums

Human Standard Serum Protein Calibrator {X0908, DAKO}

Human Standard Serum Protein High Control {X0940, Gift from DAKO}

Normal Control Rat Serum {R-7648, Sigma}

Normal Control Rat Serum {X0912, Gift from DAKO}

4.1.2 Methods

4.1.2.1 Radial Immunodiffusion (RID) Test

Initially an RID Test kit from Hycor Biomedical Inc. {Bio-Stat Diagnostics} was tried, which is based on the technique described by Feinberg [1957]. The tests were carried out according to the data sheet but the anti-human haptoglobin did not recognise the rat protein. (This kit would have been too expensive even if the anti-human haptoglobin had recognised the rat protein as only six samples could be assayed per kit.)

4.1.2.2 Rocket Immuno-electrophoresis

This method is based on the 'Laurell Rocket Technique' as described by Laurell [1966, 1972]. See Appendix 9 for protocol.

4.1.2.2.1. Polyclonal Goat Anti-Human Haptoglobin

Initially the Polyclonal Goat Anti-Human Haptoglobin Antibody from Behring was tried. This antibody did not cross react with the rat haptoglobin protein and the only sign of any reaction was a minute circular band around the well in which the rat serum had been aliquoted. The test serum used had come from rats which had been chronically dosed as long as possible with indomethacin so that the level of haptoglobin in the serum should have been very high.

4.1.2.2.2 Polyclonal Rabbit Anti-Human Haptoglobin

After an exhaustive literature search a paper was found [Persson *et al.*, 1992] which cited an anti-human haptoglobin antibody which would cross react with the equivalent rat protein [Fries & Lindström 1986]. The company which had supplied it was

Dakopatts in Denmark. The company was contacted and details were received back including the information that the antibody was available from DAKO, UK.

4.2 MYOGLOBIN - HPLC

4.2.1 Materials

4.2.1.1 Reagents

Horse Heart Cytochrome C {C-2506}, Horse Heart Myoglobin {M-1882}, Horse Skeletal Muscle Myoglobin {M-0630} and Rat Heart Cytochrome C {C-7892} from Sigma.

4.2.1.2 Solvents

Acetonitrile (Methylcyanide) HPLC Grade {UN1648, BDH or A/0627/17, Fisons} and Trifluoroacetic Acid HPLC Grade {30,203-1, Aldrich}

4.2.2 Methods

The two methods employed were an adaptation of that described by Hanson *et al.* [1992]. Myoglobin was assayed in rat serum and rat urine by reverse phase HPLC with U.V. and visible light detection using equine heart and equine muscle myoglobin as standards. The 15 cm stainless steel analytical column was packed with Chrompack P 300 RP material which is a rigid macroporous polystyrene divinylbenzene polymer with a particle size of 8 μm and a pore size of 300Å. The two mobile phases A and B were as follows, mobile phase A consisted of 0.1% (v/v) trifluoroacetic acid in acetonitrile 95:5 filtered distilled water and mobile phase B was 0.1% (v/v) trifluoroacetic acid in filtered water only. The mobile phase and glass distilled water were filtered through a 0.45 μm nylon filter {Sigma} using a glass filtering system {SPC 29, Hario} and vacuum-pump {Geryk, Alley Compressors Ltd.}. The equipment was operated at room temperature throughout.

The standards were made up in filtered distilled water as the myoglobin would not readily dissolve in the mobile phase (and even in water the highest concentration of myoglobin used, 100 $\mu\text{g/ml}$, did not go into solution quickly). Initially the serum (rat and

human) samples were diluted 1:9 with filtered distilled water and diluted 1:9 with the relevant mobile phase. However it was found that the myoglobin in the standards and the serum samples quickly deteriorated when diluted with the mobile phase. (Trifluoroacetic acid at a concentration of 0.1% (v/v) in both the water and the organic solvent results in a low pH (2) and many proteins are susceptible to unfolding in such aqueous organic solutions at acidic pH, particularly during prolonged exposure [Hanson *et al.* 1992]). Thus all standard and serum dilutions were in filtered water.

4.2.2.1 Isocratic System

Standards and rat serum samples, diluted 1:9 with filtered distilled water, were assayed using LDC components {Milton Roy, LDC Division }. The mobile phase was pumped to the column at a constant flow using a Model III Constametric pump and the samples were injected onto the column through a 100 μ l loop by injecting 250 μ l of the standard or sample into the system via an Hamilton Syringe. The eluted products were then detected using a SpectroMonitor III variable wavelength U.V. spectrophotometer and the chromatograms were produced on a chart recorder {Servogor Z10, BBC Goerz}. The mobile phase was made up and degassed by standing the bottle that contained it in a sonicator {Ultratech} for 10 minutes. (This degassing process had to be repeated on a regular basis.) The method recommended for the column by Chrompack utilised a gradient which was not possible on the isocratic system so two ratios of the mobile phases, A and B, were tried as a compromise. The ratio 50:50 was unsuccessful (the elution time of the myoglobin peak was too rapid) so the ratio utilised was 40:60, A:B, at a flow rate of 1.5 ml/min, as being the closest to the gradient conditions for the elution of myoglobin. The wavelength recommended was 220 nm, but at this wavelength the base line was very unstable and no individual peaks were obtained as proteins were seen to be eluting very close together so that the peaks overlapped. 3.0 ml of the highest myoglobin standard (100 μ g/ml) was placed in a cuvette, scanned on a spectrophotometer {SP6-500 U.V., Pye Unicam Ltd.} and a second peak was observed at a wavelength of 405 nm. Using this wavelength on the detector a flat baseline was achieved with a narrow peak eluting at around 10 minutes with the 100 μ g/ml standard.

During the course of this work it was found that levels of myoglobin in rat serum were very low so it was decided to collect urine for analysis instead as it was reported that myoglobin levels in urine were generally higher [Powell *et al.* 1884]. In addition to this urine samples are 'cleaner' than serum samples which meant that higher concentrations of sample could be passed through the column without the risk of it becoming blocked.

4.2.2.2 Gradient System

Standards and samples were assayed on a gradient system as recommended by Chrompack for the column. The mobile phase was pumped to the column at constant flow via a Constametric CM 4000 tertiary pump and programmable multiple solvent delivery system {Milton Roy LDC Division}. The mobile phase was degassed prior to use and subsequently maintained degassed by constant purging with helium {BOC}. Samples were injected onto the column through a 100 µl loop from a rotating Promis programmable autosampler {Milton Roy} and the eluted products detected using a SpectroMonitor, SM3100 variable wavelength U.V. spectrophotometer (U.V. range 190-350 nm) Chromatograms were produced and collected using a Thermochrome 737 computer system. Initially a few rat and human urine samples were run on the above system but owing to the scarcity of rat urine samples, as a consequence of a lack of success in urine collection, serum samples had to be used. However, when using a 100 µl loop, with the serum sample diluted 1:9 with filtered distilled water, the myoglobin peak was undetectable. As a result neat serum was used but the protein tended to block the column. In order to try and facilitate analysis, the serum was filtered using a micropartition system {MPS-1, Amicon} but this filtered out all the protein which had been seen to be eluting from the column.

5.0 RESULTS AND DISCUSSION - NSAIDS AND ULCER FORMATION

5.1 RESULTS - NSAIDS AND ULCER FORMATION

5.1.1 Production of Small Bowel Ulcers

5.1.1.1 Acute Oral Administration of NSAIDs

5.1.1.1.1 Animals Allowed Free Access to Food

Animals (n = 3-6) dosed with the ID₁₂₅ value of diclofenac (11.5 mg/kg), fenbufen (50.0 mg/kg), nabumetone (79.0 mg/kg), naproxen (5.0 mg/kg) and piroxicam (7.0 mg/kg) did not have any macroscopic sign of gastric or small bowel ulceration. All of the animals (n = 3), however, which had been dosed with piroxicam had dilated blood vessels coming from the mesentery (see photo. 5.1.1a) and two of the animals had very small stomachs (half the normal size) which were empty. There was also a blockage in the midileum of one of these rats which was 26 cm in length and the area involved was inflamed. Samples of this area of small bowel were taken for histological examination via light microscopy. This revealed chronic inflammation around the artery and the vein although the walls of these blood vessels appeared to be normal. The mucosa of the small bowel also appeared normal although the fat cells in the mesentery contained more cells than they would under normal conditions, some of which were lymphocytes and mast cells.

The animals (n = 4) given indomethacin ID₁₂₅ (12.5 mg/kg) exhibited ulcers in their small bowel which varied in severity from one animal which had only two discrete ulcers on the mesenteric side (0.09% of the total area) to another animal which had multiple sharp longitudinal ulceration (13.15% of the total area) accompanied by perforations and adhesions to adjacent loops of intestine. These ulcers were sharply delineated from the non-ulcerated areas of mucosa (see photo. 5.1.1b) and were accompanied by partial to complete necrosis of the muscularis externa. The longitudinal ulcers (see photo. 5.1.1c) were also on the mesenteric side of the small bowel and were positioned anywhere from the midjejunum to the proximal ileum. (There were very few, if any, ulcers in the duodenum or the distal ileum of all the animals investigated and in most animals, except in cases of severe ulceration, the ulcers were on the mesenteric side of the small bowel.) The mean percentage area of ulceration for the animals dosed with indomethacin ID₁₂₅ (n = 4) was $4.55 \pm 2.95\%$.



Photo. 5.1.1a



Photo. 5.1.1b



Photo. 5.1.1c

Photo. 5.1.1 Samples of Small Bowel Tissue from NSAID Treated Rats

a Dilated Mesenteric Blood Vessels in Tissue from a Rat Dosed with Piroxicam ID₁₂₅ Acute (7.0 mg/kg)

b Discrete Ulcers in Tissue from a Rat Dosed with Indomethacin ID₁₂₅ Acute (12.5 mg/kg)

c Longitudinal Ulcer in Tissue from a Rat Dosed with Indomethacin ID₁₂₅ Acute (12.5 mg/kg)

Samples of these ulcerated areas of small bowel were taken for histological examination via light microscopy. The longitudinal ulcers and the discrete ulcers were located along the mesentery and were full of bacteria. The discrete ulcers were usually severe, invading the muscle layer, and were associated with Peyer's patches. In these instances the necrotic tissue surrounded the Peyer's patch and often the top of the lymphoid follicle was itself necrotic. These Peyer's patches were enlarged, owing to the increase in antigenic activity, and they were full of plasma cells, lymphocytes and other mononuclear cells involved in the inflammatory response, such as neutrophils. Neutrophils were also visible on the edge of the necrotic tissue, as were macrophages. The lamina propria within the ulcerated regions was full of inflammatory cells, such as macrophages, eosinophils and mast cells. In other sections of small bowel there was some necrotic tissue immediately above the Peyer's patches, probably just involving the M cell and some of the surrounding epithelial cells, whereas the villi tissue adjacent to the Peyer's patch appeared normal. These were erosions and in these instances the Peyer's patches were active as they were enlarged and full of plasma cells, lymphocytes, neutrophils and other mononuclear cells as was the adjacent lamina propria. In the un ulcerated regions of small bowel tissue, from animals with ulcers, the Peyer's patches were normal in appearance. The lamina propria contained more inflammatory mononuclear cells than normal, a slight increase in eosinophils and macrophages, but otherwise the tissue appeared relatively normal. In tissue from animals where the discrete small bowel ulcers were more numerous, there was evidence of slight necrosis at the villus tip in regions away from Peyer's patches. Other than a few lymphocytes in a small cluster around these necrotic villi tips there were very few mononuclear cells and in the locality of these microscopic erosions there was no obvious evidence of inflammation. The mesentery associated with the ulcerated region of the small bowel was full of inflammatory cells and the fat cells were rounded owing to the numbers of mononuclear cells which had infiltrated this tissue. These cells, mostly macrophages, mast cells, lymphocytes and neutrophils, appeared to be migrating both to and from the small bowel tissue. The mesentery in the un ulcerated areas of small bowel tissue, taken from the same animals, where the Peyer's patches appeared normal contained few inflammatory cells although slightly more than the control tissue. The mesentery from control animals, however, did not have any inflammatory cells within it and as a result the fat cells were irregular in

shape. The muscle layers underlying the full thickness ulcers were also infiltrated with inflammatory cells. Small abscesses were also visible in some tissue samples along with the inflamed muscle and peritonitis. In areas where the small bowel had been perforated there was evidence of inflammation on the outside of the bowel. The Peyer's patches and villous tissue in small bowel samples taken from animals which had received an acute dose of indomethacin ID₃₀ were normal in appearance and there was no evidence of ulceration. The lamina propria, however, contained more inflammatory cells, lymphocytes and macrophages, than the normal control tissue. (The small bowel tissue from rats which had been dosed with the vehicle (10% Tween-80) was similar to that seen in control animals.)

Animals were also given acute doses of indomethacin at varying concentrations in order to determine the concentration at which ulceration commenced, and to find a dose which would reproducibly produce moderate ulceration in the small bowel. The doses of indomethacin given were the ID_{37.5}, ID₄₀, ID₅₀, ID₆₀, ID₇₀ and ID₇₅ values (3.75, 4.0, 5.0, 6.0, 7.0 and 7.5 mg/kg respectively). Animals given the ID_{37.5} (n = 3) or the ID₄₀ (n = 3) dose of indomethacin did not develop any sign of ulceration although one of the animals given the ID₄₀ dose did have one small erosion in the proximal jejunum. The animals given the ID₅₀ (n = 3) dose all had erosions (one with erosions in only the proximal jejunum and one with erosions only in the mid ileum). The one animal which displayed any ulceration had six ulcers (0.17% of the total area), the bulk of them in the mid jejunum, with erosions in the proximal jejunum and the mid ileum.

While conducting these experiments it was thought that animals purchased from Interfauna were less susceptible to NSAID toxicity than the Bath University strain of Wistar rat. As a result experiments were conducted to compare ulceration between the two strains of Wistar rats. During the second course of experiments the animals from Interfauna appeared to be more susceptible to ulceration than the Bath strain when using the ID₁₂₅ value of indomethacin as the percentage area of ulceration was greater in the Interfauna animals and there was a significant difference ($p < 0.05$) in the distribution of the ulceration (see table 5.1.1.1.1.d and fig. 5.1.1.1.1.d). The animal with the most severe ulceration also had ulcers in the antrum of the stomach which covered 45.0 mm²

Rats Given an Acute Dose of Indomethacin ID₁₂₅ (12.5 mg/kg) Killed 48 Hours Later By Cervical Dislocation

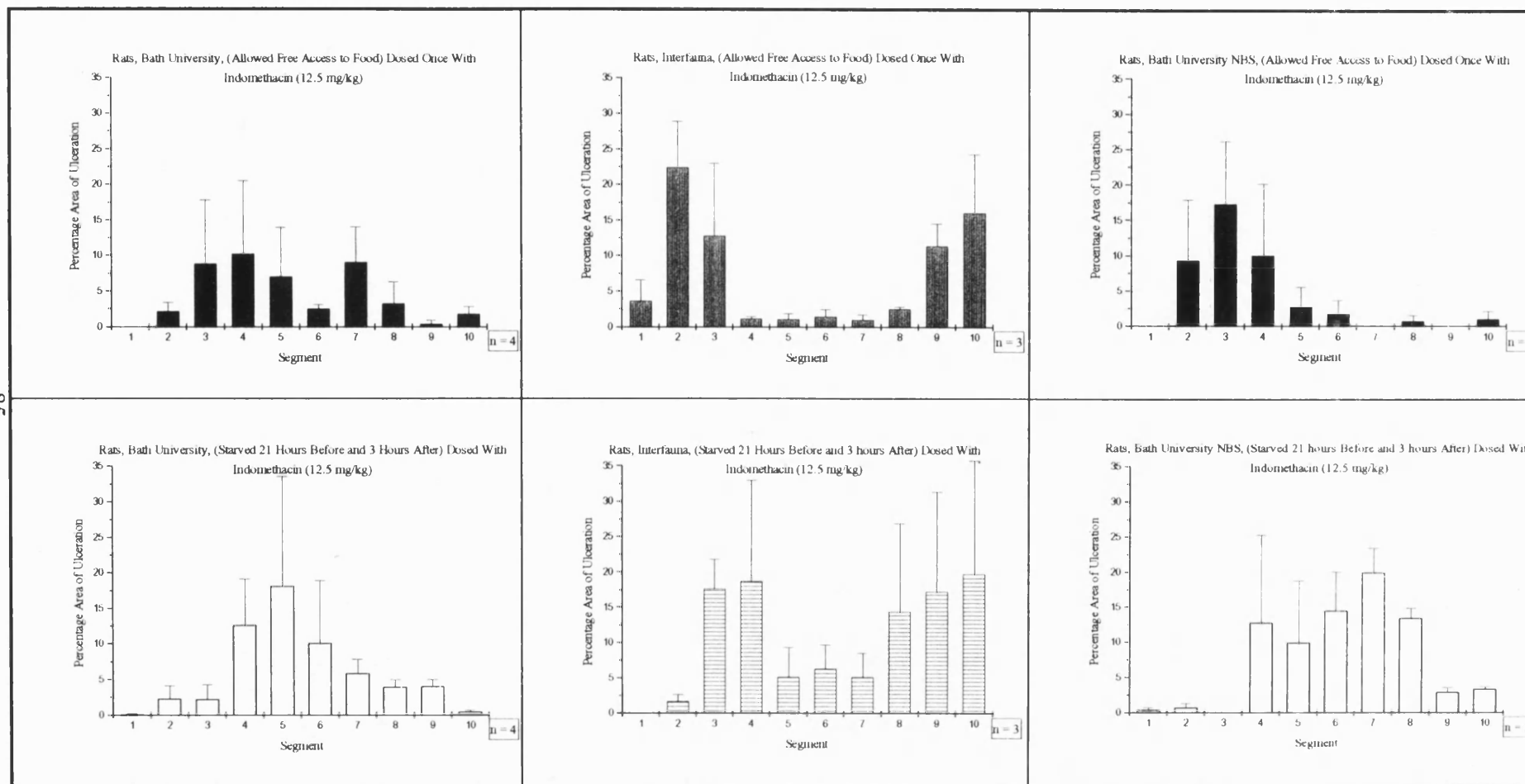


Figure 5.1.1.1d Comparison Between Interfauna and Bath University Wistar Rats (Mean \pm SEM)

of the stomach surface area. (The Bath strain were now a different group of animals as the original breeding stock had been replaced with healthier animals.) On comparing the animals after administration of the ID₇₅ value of indomethacin, however, the reverse was found. All of the animals had ulcerated small bowels but in the Bath animals (n = 3) this was statistically more severe (p < 0.05) with an area of ulceration at $7.62 \pm 2.20\%$ compared to the Interfauna animals (n = 3) which had an area of ulceration at $4.65 \pm 1.91\%$.

Wistar Rat (Strain)	Starved or Fed Prior to Dosing	Mean Total Percentage Area of Ulceration (%)
Bath University	Fed	4.55 ± 2.95
Bath University	Starved	5.94 ± 2.34
Interfauna	Fed	7.36 ± 2.18
Interfauna	Starved	10.63 ± 4.21
Bath University NBS	Fed	4.66 ± 1.56
Bath University NBS	Starved	7.75 ± 2.16

Table 5.1.1.1.1.d Comparison Between Interfauna and Bath University Wistar Rats Given an Acute Dose of Indomethacin ID₁₂₅ (12.5 mg/kg) (Mean \pm SEM)

The ulceration in the small bowels of the animals (n = 3) dosed with indomethacin ID₇₀ was less severe than that in the animals (n = 3) given the ID₇₅ dose although one of the animals did have two of the longitudinal ulcers in the midjejunum of approximately 1.0 to 1.5 cm in length (mean surface area $1.63 \pm 0.39\%$). As a result it was decided to use a dose of indomethacin at a concentration of ID₆₀ for the rest of the experiments as at this concentration ulceration in the small bowel was moderate.

During the course of these experiments, however, it was obvious that there was a lot of variation between and within groups of animals as to their susceptibility to NSAID toxicity and the resulting severity of ulceration. As a result it was decided that, during all experiments, there would have to be a control group of animals, dosed with indomethacin ID₆₀, that could be used as a direct comparison between it and other drug regimes. Within seven groups (n = 3) of Bath animals, given a dose of indomethacin ID₆₀, there was in fact a statistical difference (p < 0.05) in the degree of ulceration between most of the groups with the total area of ulceration ranging from $0.31 \pm 0.06\%$ to $5.6 \pm 1.62\%$.

5.1.1.1.2 Animals Starved Before and After NSAID Administration

Animals (n = 3-6) dosed with the ID₁₂₅ value of diclofenac (11.5 mg/kg), fenbufen (50.0 mg/kg), nabumetone (79.0 mg/kg), naproxen (5.0 mg/kg) and piroxicam (7.0 mg/kg) did not display any macroscopic sign of small bowel ulceration.

The original Bath University rats (n = 4) dosed with indomethacin ID₁₂₅ (12.5 mg/kg) all displayed small bowel ulceration and the severity was greater in these animals than seen in those which had been allowed free access to food before and after dosing (see table 5.1.1.1.1.d and fig. 5.1.1.1.1.d). Similarly, this experiment was repeated comparing the Bath strain (NBS) of animals with those from Interfauna. As with the animals, in the parallel experiment, allowed free access to food; the percentage area of ulceration was greater in the Interfauna animals when compared the Bath strain (NBS) ($10.63 \pm 4.21\%$ as opposed to $7.75 \pm 2.16\%$) and there was a significant difference ($p < 0.05$) in the distribution of the ulceration (see table 5.1.1.1.1.d and fig. 5.1.1.1.1.d). Two (n = 3) of the Interfauna animals had ulcers in the antrum of the stomach, covering a total surface area of 35.5 mm² and 23.0 mm² respectively, although in this group the animal with the most stomach ulcers had the least ulceration in the small bowel. The overall ulceration was more severe in the animals starved before and after dosing with indomethacin ID₁₂₅ than in the equivalent strains of Wistar rats allowed free access to food and water. Conversely, as with the animals allowed free access to food and water before and after dosing, the degree of ulceration in the animals given the ID₇₅ dose was least in the Interfauna animals and whereas all the Bath animals (n = 3) had ulcers in the small bowel, two of the Interfauna animals (n = 3) had no sign of ulceration at all. (Total mean percentage area of ulceration for the Bath animals was $2.89 \pm 0.97\%$ compared to the Interfauna animals with a mean percentage area of ulceration of $0.11 \pm 0.11\%$). There was also a statistical difference in the degree of ulceration ($p < 0.05$) with the starved animals given ID₇₅ dose having fewer ulcers than the animals allowed free access to food and water before and after dosing (see table 5.1.1.1.2 and fig. 5.1.1.1.2).

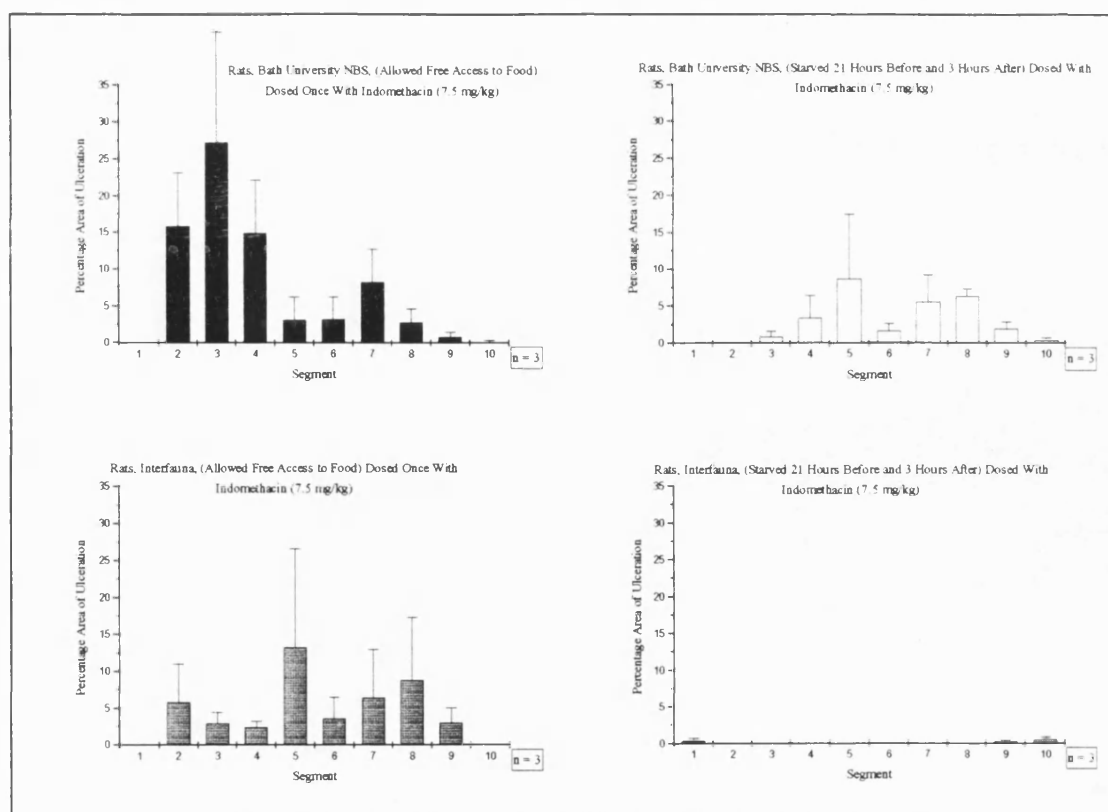


Figure 5.1.1.1.2 Comparison Between Interfauna and Bath University Wistar Rats Given an Acute Dose of Indomethacin ID₇₅ (7.5 mg/kg) (Mean \pm SEM)

Wistar Rat (Strain)	Starved or Fed Prior to Dosing	Mean Total Percentage Area of Ulceration (%)
Bath University 2	Fed	7.62 \pm 2.20
Bath University 2	Starved	2.89 \pm 0.97
Interfauna	Fed	4.65 \pm 1.91
Interfauna	Starved	0.11 \pm 0.11

Table 5.1.1.1.2

This group of animals (Interfauna/Bath University) dosed with indomethacin ID₇₅ had all weighed around 325 g when dosed (the Interfauna animals had been supplied at a greater weight than requested so that many of them exceeded 250 g before they could be used experimentally). Consequently the unexpected results of the animals dosed with indomethacin ID₇₅, when compared to the animals dosed with indomethacin ID₁₂₅, (which was the reverse) was thought to be weight related. As a result groups of rats (Bath University NBS) of differing weights (150, 250, 350 and 450 g) were given an acute dose of indomethacin ID₇₅ (n = 3) to ascertain whether the dosed weight had an

effect on indomethacin-induced ulceration. The animals which had weighed approximately 250 g when dosed had the least ulceration but overall there was no significant difference within the groups.

5.1.1.1.3 Correlation Between Indomethacin Acute ID value and Area of Ulceration

There was a significant correlation ($r = 0.57$, $p < 0.001$) between the acute dose of indomethacin administered and the total percentage area of ulceration seen in the small bowel of rats ($n = 92$) as measured by regression analysis (see fig 5.1.1.1.3).

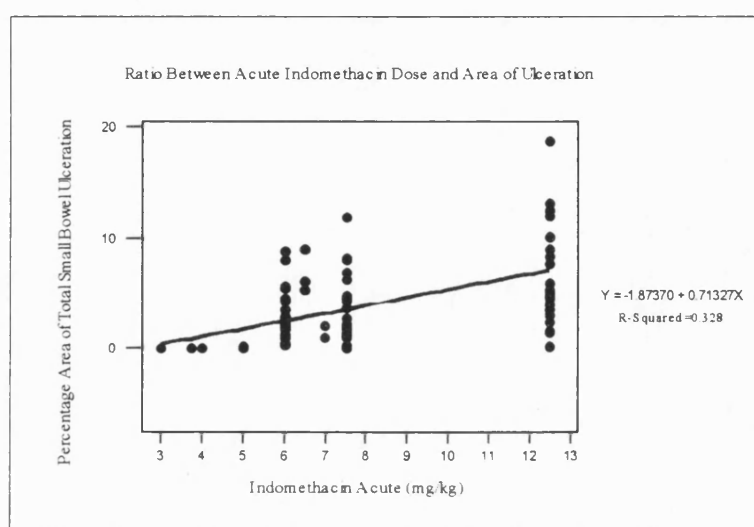


Figure 5.1.1.1.3 Ratio Between Acute Indomethacin Dose and Area of Ulceration

5.1.1.2 Chronic Oral Administration of NSAIDs

5.1.1.2.1. Short Term Dosing

Initially no ulcers were found in any of the animals ($n = 3$) dosed chronically, for seven days, with the ID_{25} values of the drugs diclofenac (2.3 mg/kg), fenbufen (10.0 mg/kg), indomethacin (2.5 mg/kg) nabumetone (15.8 mg/kg), naproxen (1.0 mg/kg) and piroxicam (1.4 mg/kg). However, when the indomethacin experiment was repeated using the ID_{25} value the chronic dosing had to be halted after 5 days because two of the rats began to lose weight. The mean percentage area of ulceration ($0.25 \pm 0.12\%$) was significantly higher in this group ($p < 0.05$) although one animal did not have any sign of ulceration. The initial group of animals had been purchased from Interfauna whereas the

second group of animals were of the Bath University strain of Wistar rats. As a result this experiment was repeated, comparing the two strains of animals. On this occasion only one animal from each group ($n = 3$) displayed any form of ulceration, the Interfauna animal being more ulcerated than the animal from the Bath University strain (the new breeding stock, NBS) although this was not significant (mean percentage area of $0.01 \pm 0.01\%$ compared to $0.22 \pm 0.22\%$).

Animals (Bath University, NBS) were treated for 7 days with indomethacin $ID_{27.5}$ and killed on day ten. The total percentage area of ulceration for these animals ($0.08 \pm 0.02\%$) was greater than the ulceration found in the same strain of Bath animal similarly dosed with the ID_{25} value of indomethacin ($0.01 \pm 0.01\%$) although overall this was not significant ($p = 0.6$). The distribution of the ulceration, however, was significantly different ($p < 0.05$) with most of the ulcers appearing in the jejunum.

Animals ($n = 3$) from the original Bath University strain of Wistar rats had been treated with indomethacin ID_{50} but only for three days as one of the animals had begun to lose weight after the third dose (mean percentage area $2.74 \pm 1.26\%$). Consequently the dose was reduced and rats ($n = 3$) from the Bath University strain (again the original breeding stock) were dosed for 5 days with indomethacin $ID_{37.5}$. These animals also had to be killed early (on day 6) as one of these animals had begun to lose weight (mean percentage area of ulceration $2.02 \pm 0.94\%$). (These animals were significantly ($p < 0.05$) more ulcerated than the same batch of animals dosed for 5 days with the ID_{25} value of indomethacin.) It was a consequence of the level of ulceration found in these animals that the original experiment with the ID_{25} value of indomethacin was repeated as the severity of ulceration at the $ID_{37.5}$ dose had been unexpected owing to the fact that initially no ulceration had been seen after the initial indomethacin ID_{25} chronic dosing experiment (performed on the original batch of Interfauna animals).

Animals (Bath University, NBS) were also treated with indomethacin ID_{30} chronically, one group for three days and one group for six days, with the animals all being killed on the seventh day. Only one of the animals dosed for three days had any sign of ulceration and this was one small ulcer (0.03% of the small bowel area) in the midjejunum. The

animals which had been treated with indomethacin ID₃₀ for six days all had ulceration of the small bowel, which was significantly greater than that of the animals treated for three days ($p < 0.05$) with most of the ulcers being located within the jejunum ($0.88 \pm 0.48\%$ of the total surface area). Animals (Bath University, NBS) were then treated with indomethacin ID₄₀ for three days and killed on day seven. In this group one of the animals did not have any sign of ulceration and of the animals that did, the majority of ulcers occurred between the midjejunum and the midileum. The total area of ulceration ($0.13 \pm 0.12\%$) was not significantly greater however. This experiment was repeated using the ID₄₅ value of indomethacin but the experiment had to be terminated after three days when one of the three animals (Bath University, NBS) began to lose weight after only two doses. In this instance the ulceration stretched from the proximal jejunum to the distal ileum and there were many longer ulcers present (although nothing similar to the longitudinal ulceration seen with larger doses of indomethacin). The area of ulcerated small bowel in these animals ($3.84 \pm 1.68\%$) was significantly greater ($p < 0.05$) than that seen in the animals dosed with indomethacin ID₄₀ for three days (see fig. 5.1.1.2.1).

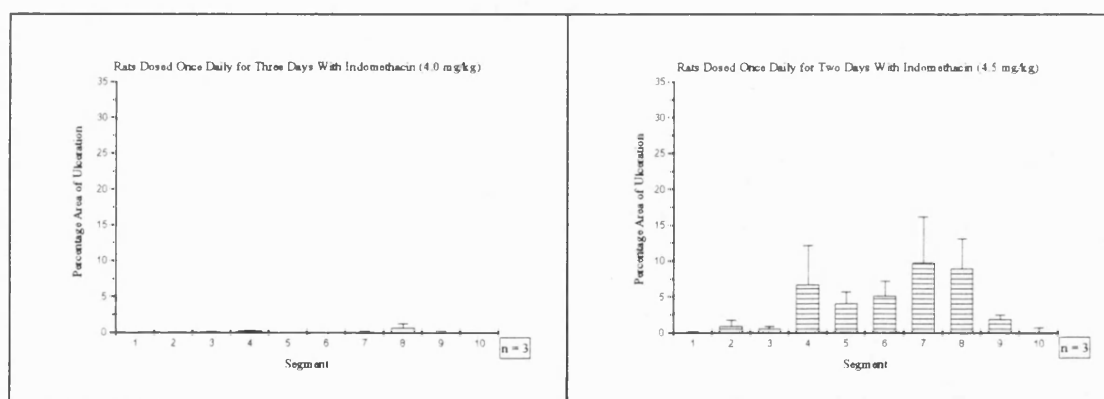


Figure 5.1.1.2.1 Comparison Between Animals Dosed Chronically With Either Indomethacin ID₄₀ (4.0 mg/kg) or Indomethacin ID₄₅ (4.5 mg/kg) (Mean \pm SEM)

Animals (Bath University strain) were dosed with the ID₁₂₅ value of all the drugs, except for indomethacin, for seven days and killed on day 10. Animals ($n = 3$) dosed with the ID₁₂₅ value of fenbufen (50.0 mg/kg), nabumetone (79.0 mg/kg), naproxen (5.0 mg/kg) and piroxicam (7.0 mg/kg) did not have any sign of gastric or small bowel ulceration.

The animals dosed with piroxicam did have dilated blood vessels coming from the mesentery in all the animals but there was no sign of inflammation visible within the small bowel. The result obtained with piroxicam was unexpected as other researchers had obtained ulceration in rats using this drug under similar circumstances [Spangler, 1993]. As a result this experiment was repeated at a later date, this time using Bath University Wistar rats from the new breeding stock, but as before there was no sign of ulceration. (The mucosal surface in some areas of the jejunum, however, was quite fragile.)

The animals ($n = 3$) dosed with diclofenac ID_{125} (11.5 mg/kg) had to be killed on the fifth day, after they had only received four doses, as one of the rats had begun to lose weight and the small bowel of this animal was too badly ulcerated for ulcer analysis. The remaining animals had few ulcers ($0.64 \pm 0.64\%$ of the total surface area) but the degree of ulceration was severe. Chronic dosing was repeated using the ID_{75} , ID_{100} and $ID_{112.5}$ values of diclofenac (6.9, 9.2 and 10.35 mg/kg respectively). The animals ($n = 3$) dosed with the ID_{75} value of diclofenac for 7 days did not have any form of ulceration at all and in the group ($n = 3$) given the ID_{100} value chronically, one of the rats also had no form of ulceration (mean percentage area $0.06 \pm 0.04\%$). (In this group of animals the ulcers were in the proximal jejunum and in the midileum only.) The animals ($n = 3$) given the $ID_{112.5}$ value of diclofenac were only give 4 doses, as one of the rats had begun to lose weight, and were killed on the sixth day (mean percentage area of ulceration $1.31 \pm 0.26\%$). Ulceration among this group of animals ranged from the proximal jejunum to the mid ileum and consisted mostly of discrete ulcers except that all the animals had one extremely large ulcer ($70.0 \pm 12.5 \text{ mm}^2$) at the beginning of the fifth segment which was part of the distal jejunum.

5.1.1.2.2. Long Term Dosing

Groups of four rats were dosed orally, once daily, for 21 days, over a four week period, with the ID_{125} values of fenbufen (50.0 mg/kg), nabumetone (79.0 mg/kg), naproxen (5.0 mg/kg) and piroxicam (7.0 mg/kg). No visible sign of ulceration was found in either the stomachs or the small bowels of these animals although during the course of the experiment it was obvious that the groups of animals were gaining weight at a different rate. The mean weight gain between the four groups was not significantly different when

comparing the four treatments simultaneously, however, the animals given fenbufen ID₁₂₅ weighed significantly less ($p < 0.05$) than the animals dosed with naproxen ID₁₂₅ (185.00 ± 8.10 g versus 140.00 ± 20.08 g) when calculating the least significant difference (L.S.D._(0.05)) between a pair of treatments. The mean weight gain for the animals given nabumetone was 166.25 ± 6.10 g and for the animals given piroxicam it was 161.25 ± 4.80 g. There was no significant difference in the lengths of the small bowels between the groups, the mean lengths were as follows, piroxicam 119.75 ± 3.88 cm, nabumetone 116.00 ± 1.37 cm, naproxen 114.50 ± 0.83 cm and fenbufen 111.75 ± 1.24 cm.

5.1.1.3 Pretreatment With Antibiotics

5.1.1.3.1 Individually

Groups of rats ($n = 3$) were dosed orally, once daily, for three days with one of the six antibiotics selected, amoxycillin (50 mg/kg), cloxacillin (100 mg/kg), erythromycin (100 mg/kg), metronidazole (100 mg/kg), nalidixic acid (200 mg/kg) and neomycin sulphate (100 mg/kg). The rats were then given an acute dose of indomethacin ID₆₀ 24 hours after the final dose of antibiotic and then killed 48 hours after the dose of indomethacin.

Pretreatment for Three Days Prior to Acute Dose of Indomethacin ID ₆₀	Drug Concentration (mg/kg)	Mean Percentage Area of Total Ulceration
Distilled Water	0	5.60 ± 1.62
Metronidazole	100	0.29 ± 0.05
Amoxycillin	50	0.45 ± 0.17
Distilled Water	0	3.13 ± 1.25
Neomycin Sulphate	100	2.11 ± 0.37
Cloxacillin	100	0.15 ± 0.09
Distilled Water	0	1.97 ± 0.49
Nalidixic Acid	200	5.21 ± 2.87
Erythromycin	100	4.72 ± 1.76

Table 5.1.1.3.1 Comparison Between Animals Pretreated with Antibiotics or Distilled Water (Mean \pm SEM)

The animals pretreated with amoxycillin, cloxacillin and metronidazole had significantly ($p < 0.05$) less ulceration than the control animals which had only been pretreated with distilled water prior to the indomethacin challenge (see table 5.1.1.3.1). The animals pretreated with cloxacillin and metronidazole had a few ulcers in the proximal jejunum but no ulceration in the rest of the small bowel. The animals pretreated with amoxycillin

Rats Pretreated for Three Days With Either Distilled Water or Antibiotics Prior to an Indomethacin ID₆₀ (6.0 mg/kg) Challenge

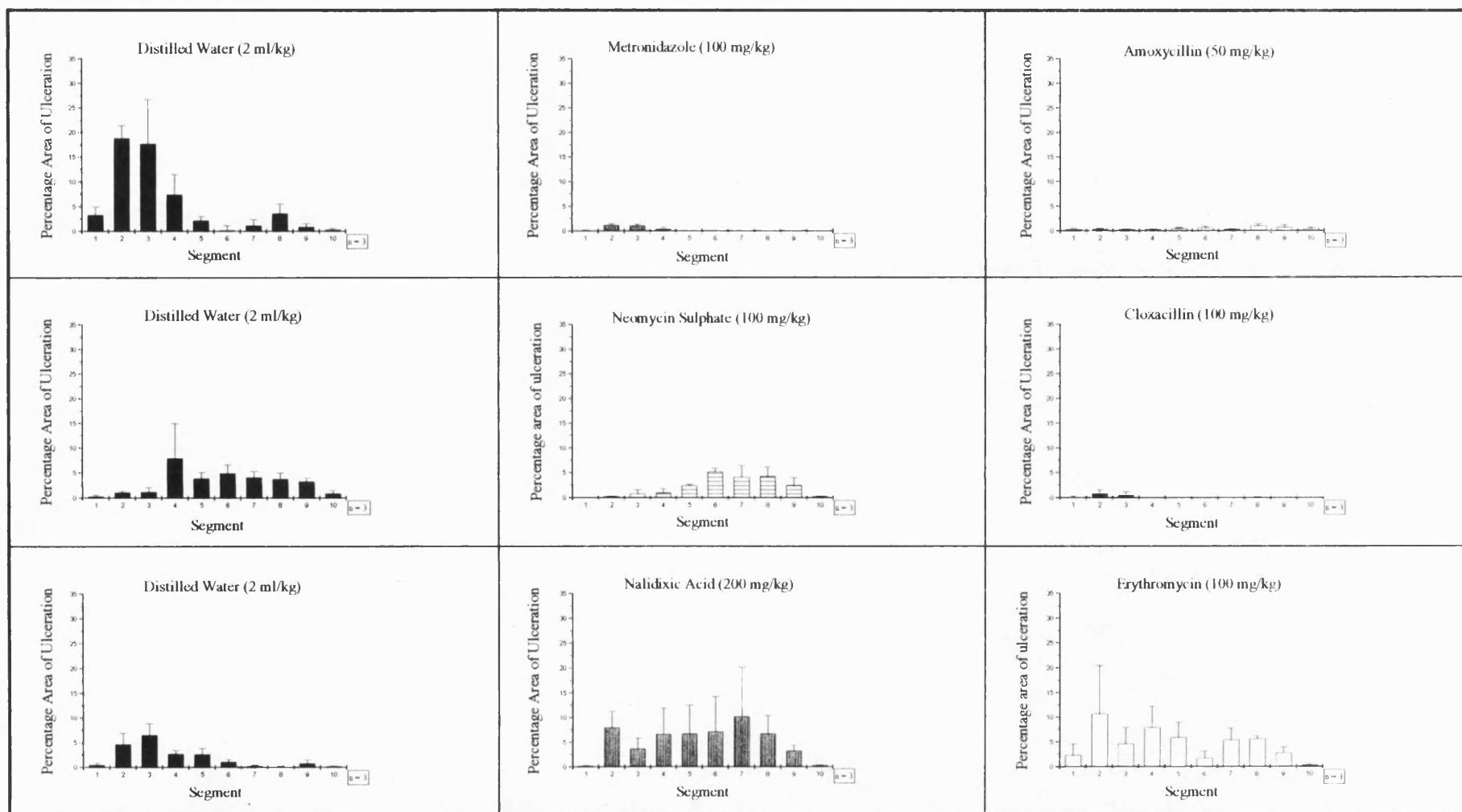


Figure 5.1.1.3.1 Comparison Between Animals Pretreated with Antibiotics or Distilled Water (Mean ± SEM)

had a few ulcers distributed from the proximal jejunum to the distal ileum, where the ulcer ratio was slightly higher (see fig. 5.1.1.3.1).

The animals pretreated with erythromycin and nalidixic acid had significantly ($p < 0.05$) more ulceration than the control animals (see table 5.1.1.3.1), the ulceration in the erythromycin group was greater in the first half of the small bowel whereas the animals in the nalidixic acid group had more ulcers in the latter half of the small bowel (see fig. 5.1.1.3.1).

No significant difference in ulceration was found between the animals pretreated with neomycin sulphate and the control animals although the total percentage area of ulceration for the neomycin sulphate group ($2.11 \pm 0.37\%$) was lower than that of the control animals ($3.13 \pm 1.25\%$). The majority of ulcers in the neomycin sulphate group were in the latter half of the small bowel (see fig. 5.1.1.3.1).

5.1.1.3.2 Combined

No visible sign of ulceration was found at all in rats pretreated for three days with a combination of metronidazole (100 mg/kg) and amoxycillin (50 mg/kg) followed 24 hours later with an indomethacin challenge (either ID₆₀, ID₆₅ or ID₇₀). The ulceration was less than that seen in the control animals (pretreated with distilled water) when the three day antibiotic pretreatment occurred seven days prior to the indomethacin ID₆₀ challenge. The ulceration was still less than that seen in the control animals when the three day antibiotic pretreatment was given 14 days prior to the indomethacin challenge although the distribution of ulceration was beginning to mirror that seen in the control animals (see fig. 5.1.1.3.2). There was a significant difference ($p < 0.05$) in the degree of ulceration between the four groups.

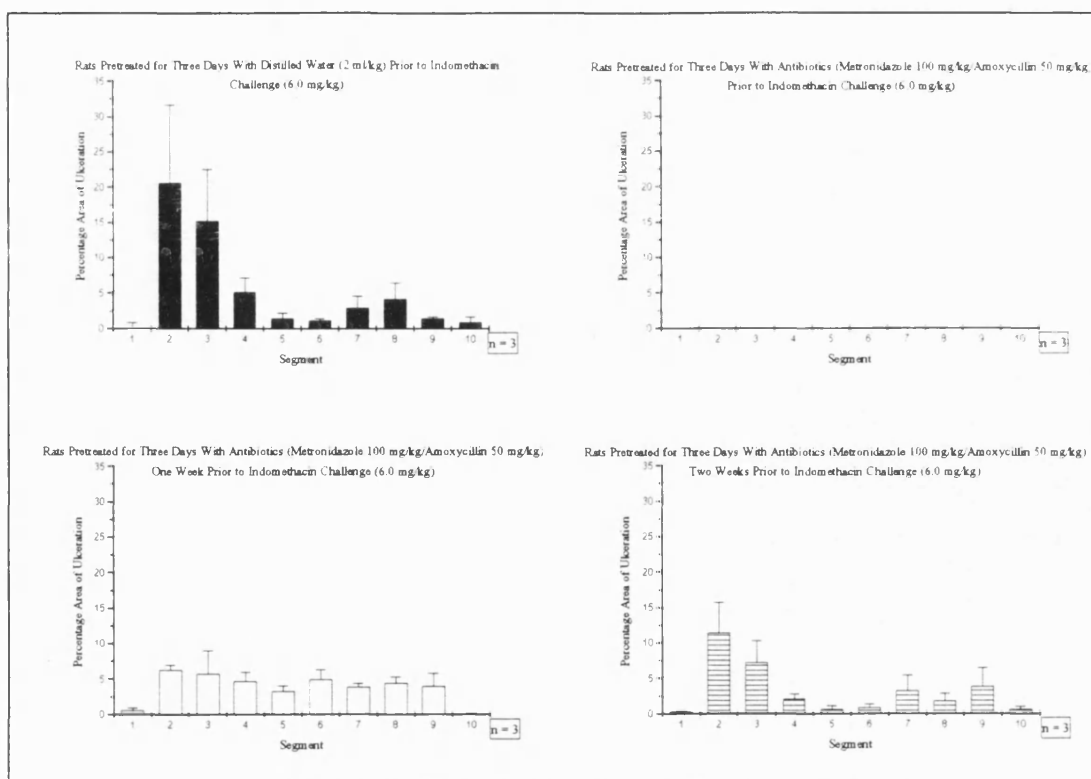
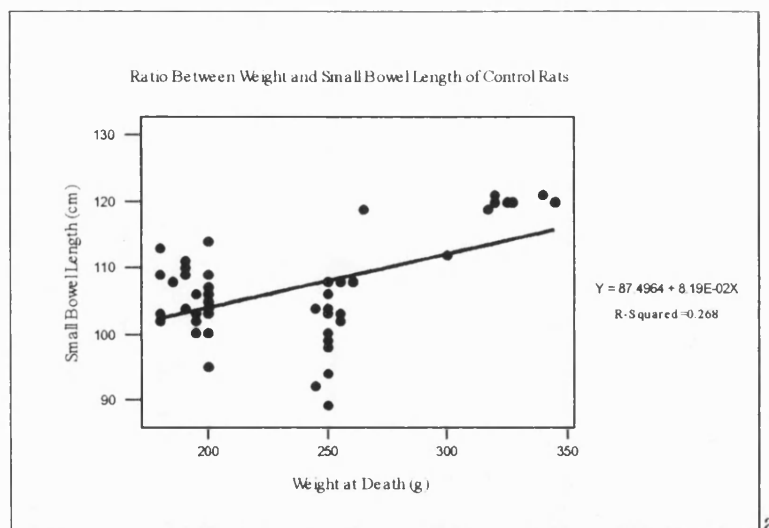


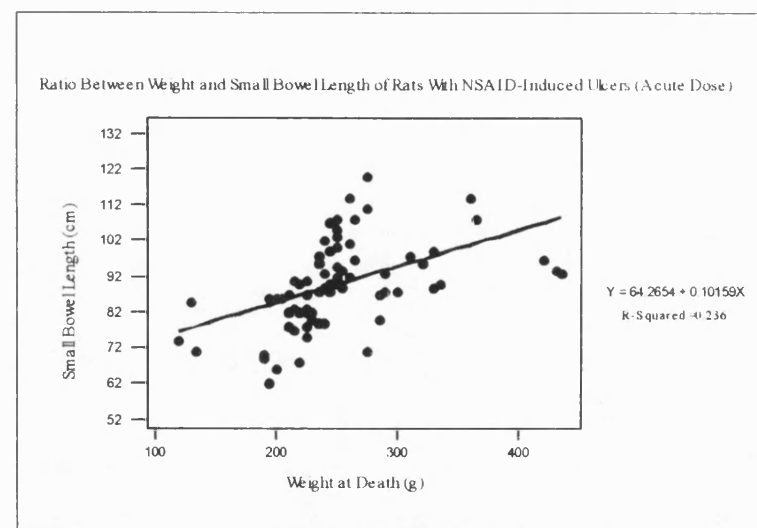
Figure 5.1.1.3.2 Comparison of Ulceration Between Rats Pretreated With a Combination of Antibiotics (Metronidazole 100 mg/kg/Amoxycillin 50 mg/kg) Two Weeks, One Week and Immediately Prior to an Indomethacin ID₆₀ (6.0 mg/kg) Challenge (Mean \pm SEM)

5.1.1.4 Correlation Between Weight at Death and Small Bowel Length

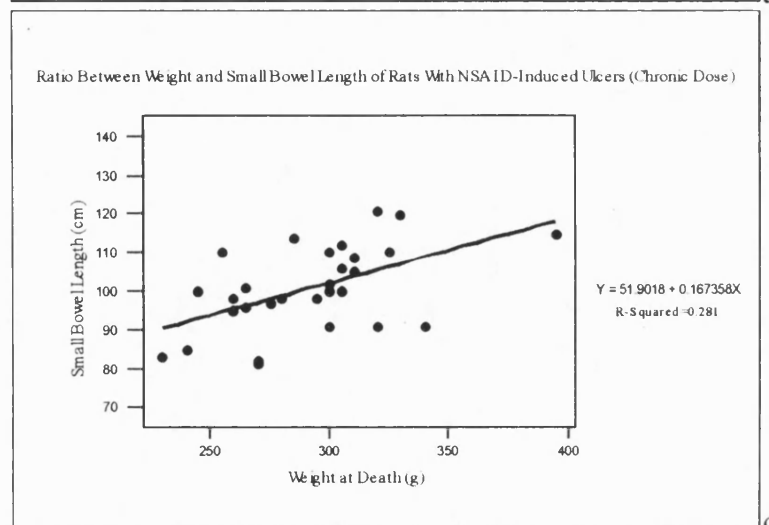
Animals which had small bowel ulceration after an acute dose of indomethacin had a shorter small bowel than control animals which had not been given NSAIDs. In the control rats ($n = 50$) there was a significant correlation ($r = 0.52$, $p < 0.001$) between the weight of the animal and the small bowel length as measured by regression analysis (see fig. 5.1.1.4a). There was also a significant correlation ($r = 0.49$, $p < 0.001$) between the weight at death and the length of the small bowels of animals ($n = 76$) with indomethacin acute ulceration (see fig. 5.1.1.4b). There was no correlation ($r = 0.1$), however, between the weight at death and the length of the small bowel of animals ($n = 43$) which had been given an acute dose of an NSAID but had not developed any form of ulceration.



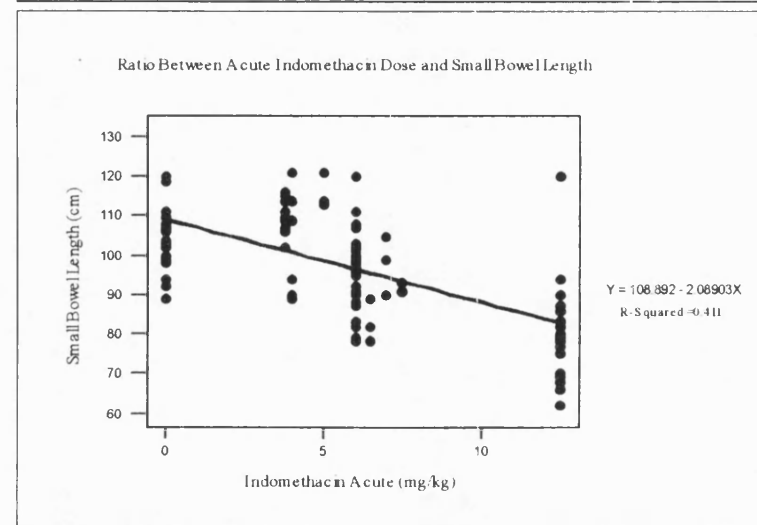
a



b



c



d

Figure 5.1.1.4 Correlation Between Weight at Death and Small Bowel Length

Comparing animals which had been given NSAIDs chronically there was a significant correlation ($r = 0.53$, $p < 0.01$) between the weight at death and the length of the small bowels of animals ($n = 29$) which had NSAID-induced ulceration (see fig. 5.1.1.4c). In this instance there was also a significant correlation ($r = 0.38$, $p < 0.001$) between the weight at death and the small bowel length of animals ($n = 63$) dosed chronically with NSAIDs which had not developed any ulceration.

Surprisingly there was a greater correlation between the weight at death and the small bowel length of animals pretreated with antibiotics before the acute dose of indomethacin. In the animals ($n = 36$) which developed ulcers after pretreatment with antibiotics the correlation ($r = 0.66$) was significant ($r > 0.52 \equiv p < 0.001$) and similarly in the animals ($n = 12$) which did not develop any ulcers the correlation ($r = 0.69$) was significant ($r > 0.66 \equiv p < 0.01$).

5.1.1.5 Correlation Between Acute Indomethacin Dose and Small Bowel Length

There was a good correlation ($r = 0.64$) which was significant ($r > 0.32 \equiv p < 0.001$) between the small bowel length of animals ($n = 103$) and the acute dose of indomethacin administered, as measured by regression analysis (see fig. 5.1.1.4d).

5.1.1.6 Measurement of Small Bowel pH

The stomach contents of all the control rats investigated ($n = 6$) were found to be at pH 3.0. However, immediately beyond the pyloric sphincter, the small bowel was found to have a pH of 7.0 until the distal ileum where the pH began to rise approximately 32 cm prior to the ileocaecal junction (see fig. 5.1.1.6). These pH values were used to calculate, using the Henderson-Hasselbalch equation, the gut/plasma ratios within the small bowel of the NSAIDs used during this project (see table 5.1.1.6).

Henderson-Hasselbalch equation:-

$$R_{\text{gut/plasma}} = \frac{1 + 10^{(\text{pH}_g - \text{pK}_a)}}{1 + 10^{(\text{pH}_p - \text{pK}_a)}}$$

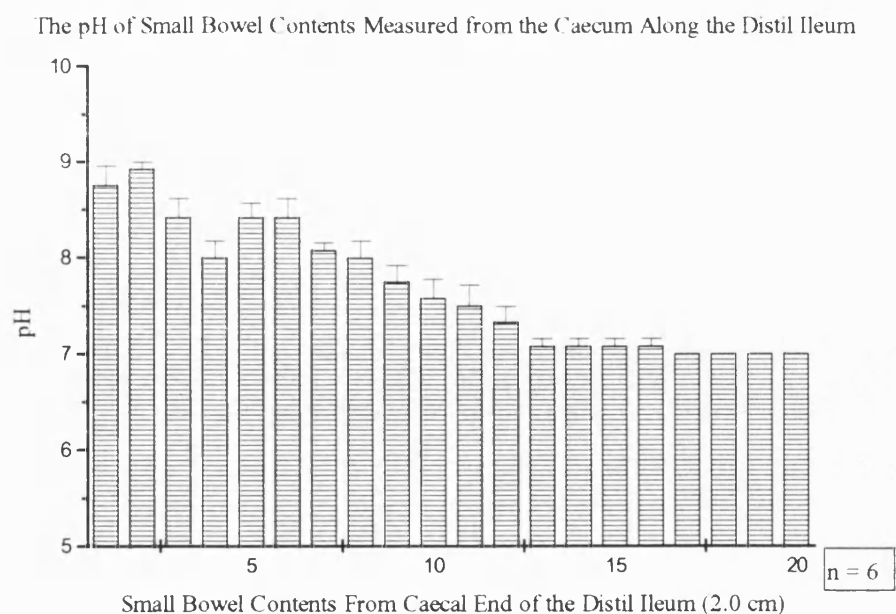


Figure 5.1.1.6 Distribution of pH in the Terminal Ileum (Mean ± SEM)

Ileal Segments From Caecal End	Small Bowel Contents pH	NSAID pKa				
		Diclofenac 3.9	Naproxen 4.2	Indomethacin 4.5	Fenbufen 5.7	Piroxicam 6.3
1	8.75	22.38	22.37	22.36	21.97	20.81
2	8.92	33.10	33.09	33.07	32.48	30.75
3	8.42	10.47	10.47	10.46	10.29	9.77
4	8	3.98	3.98	3.98	3.92	3.76
5	8.42	10.47	10.47	10.46	10.29	9.77
6	8.42	10.47	10.47	10.46	10.29	9.77
7	8.08	4.79	4.78	4.78	4.71	4.51
8	8.00	3.98	3.98	3.98	3.92	3.76
9	7.75	2.24	2.24	2.24	2.21	2.15
10	7.58	1.51	1.51	1.51	1.50	1.48
11	7.5	1.26	1.26	1.26	1.25	1.24
12	7.33	0.85	0.85	0.85	0.85	0.86
13	7.08	0.48	0.48	0.48	0.49	0.52
14	7.08	0.48	0.48	0.48	0.49	0.52
15	7.08	0.48	0.48	0.48	0.49	0.52
16	7.08	0.48	0.48	0.48	0.49	0.52
17	7	0.40	0.40	0.40	0.41	0.44
18	7	0.40	0.40	0.40	0.41	0.44
19	7	0.40	0.40	0.40	0.41	0.44
20	7	0.40	0.40	0.40	0.41	0.44

Table 5.1.1.6 Predicted Gut/Plasma Ratio of NSAIDs Along the Small Bowel

5.1.2 Isolation of Small Bowel Flora Associated With NSAID Induced Lesions.

5.1.2.1 Tissue Scrapings From Ulcer Base

Agar	Source	Description	Bacterium	Identification (%)
McConkey 3	Pink Colony	Gram -ve rods	<i>Escherichia coli</i>	99.1
McConkey 3	Pink Colony	Gram -ve rods	<i>Escherichia coli</i>	99.1
McConkey 3	Opaque Colony	Gram -ve rods	<i>Proteus mirabilis</i>	99.9
Nutrient	Spreading Colony	Gram -ve rods	<i>Proteus mirabilis</i>	99.9
Nutrient	Pink Colony	Gram -ve rods	<i>Escherichia coli</i>	99.1

Table 5.1.2.1 Bacteria Isolated and Identified from Tissue Scrapings from Ulcer Base

5.1.2.2 Complete Ulcers Excised Aerobically

Large gram-positive cocci were also isolated from one of the Blood Agar plates but this bacterium has yet to be identified. The only bacterium isolated via the anaerobic jars was a type of *Escherichia coli* which is a facultative anaerobe. As a result it was decided that ulcers would have to be excised anaerobically in order to try and isolate all the microorganisms which had been visualised and tentatively identified via scanning electron microscopy (see photos. 5.1.3 and 5.1.4).

Agar	Source	Description	Bacterium	Identification (%)
Deoxycholate Citrate	Dark Pink Colony	Gram -ve rods	<i>Escherichia coli</i>	85.4
Deoxycholate Citrate	Pale Pink Colony	Gram -ve rods	<i>Proteus mirabilis</i>	99.9
Deoxycholate Citrate	Dark Pink Colony	Gram -ve rods	<i>Escherichia coli</i>	85.4
Blood	Dark Black Colony	Gram -ve rods	<i>Escherichia coli</i>	99.9
Blood	Spreading Colony	Gram -ve rods	<i>Salmonella</i>	?

Table 5.1.2.2 Bacteria Isolated and Identified Complete Ulcers Excised Aerobically

Cooked Meat	Isolate (1)		Isolate (2)		Isolate (3)		Mixture 1A		Mixture 2A	
Antibiotic	Aerob (mm)	Anaerob (mm)	Aerob (mm)	Anaerob (mm)	Aerob (mm)	Anaerob (mm)	Aerob (mm)	Anaerob (mm)	Aerob (mm)	Anaerob (mm)
Amoxycillin AML 2	1.5	—	2.0	—	0.5	—	3.5	—	1.5	—
Amoxycillin AML 10	3.5	2.0 C	4.5	—	5.5 H	—	6.0	—	4.0 SH	—
Amoxycillin AML 25	4.0	7.0 C	7.0 CO	—	0	0	9.5	4.0 C	5.0 HOR	—
Metronidazole MTZ 5	0	—	0	—	0	0	0	0	0	—
Metronidazole MTZ 50	0	—	0	—	0	0	0	0	0	—
Cloxacillin OB 5	0	—	0	—	0	0	0	0	0	—
Chloramphenical C 10	5.5 H	4.5	4.0	3.5	2.5 H	0	5.0 CO	5.5	3.5	3.5
Neomycin N 30	5.5 CO	2.0	5.0 C	—	5.5	0	8.0 HOR	0	3.0 H	—
Ampicillin AMP 2	1.5	2.0	1.5 H	—	2.0 H	0	1.5 H	—	1.5	—
Gentamicin CN 10	5.5	0	6.0	—	4.0	0	1.5	0	5.5	—
Penicillin G P2 (v)	0	—	0	—	0.5 H	0	1.5	—	0	—
Erythromycin E 10	0	0	0	—	0	0	0	0	0	—
Trimethoprim W 1.25	3.5 C	0	0	—	2.0 H	0	3.5 VH	0	0	—
Cephadrine CE 30	4.5 C	4.0 C	3.5 C	—	3.5	—	4.0 HOR	—	3.5 HOR	—
Cefuroxime CXM 30	5.5	5.0	4.5 SH	—	6.0 SH	6.5	7.0 H	—	4.5 H	—
Amoxycillin Clavulanic Acid AMC 30	4.5	4.5	4.0	—	5.0 HOR	—	6.0	—	4.5	—
Ciprofloxacin CIP 1	9.5 CO	2.0 C	10.5 CO	—	10.5 HOR	—	12.0 HOR	0.5 C	9.5 HOR	—
Cephalexin CL 30	1.0 C	0	3.5 HC	—	3.0	—	3.5 H	—	0.5 H	—
Nalidixic Acid NA30	5.0 C	4.0 C	4.5 C	—	4.5	3.5	5.0 VH	2.0 VHC	4.5 H	—
Ciprofloxacin CIP 5	10.5	9.5	10.0 HOR	9.5	13.5 HOR	9.5	5.5 H	5.5 C	6.0 H	—
Oxytetracycline OT 30	5.0 C	4.5	3.5 H	2.5 H	0	0	0	2.0	6.5 VH	—
Sulpha- methoxazole RL25	0	0	0	—	4.0 H	—	0	0.5	0	—
Amikacin AK 10	4.5 C	0	4.5 C	—	2.0	—	4.5 VH	0.5	4.0 H	—
Aztreonam AM 30	9.0 C	1.5	0	—	0	0	0	0	7.0 VH	—

Table 5.1.2.4a Results of Antimicrobial Susceptibility Test of Microorganisms Cultured in Cooked Meat Broth

5.1.2.3 Complete Ulcers Excised Anaerobically

The only bacterium isolated via this procedure was again a type of *Escherichia coli* which is a facultative anaerobe. At this juncture it was decided that in order to isolate obligate anaerobes, such as *Bacteroides* and *Fusobacterium*, which were suspected as being some of the bacteria with a role in the ulcerative process, the ulcers would have to be excised in a totally oxygen free environment. The Atmosbag had not proved to be suitable for this purpose as it was very difficult to excise the ulcers under such circumstances. As a result a glove box was built and an oxygen trap was procured so as to ensure that the oxygen free nitrogen gas was completely free of any oxygen.

The bacteria isolated via this procedure were to be investigated to determine whether or not they were capable of performing chemical biotransformations *in vitro*. Unfortunately Dr Colin Soper, the supervisor for the microbiological aspects of this project, died suddenly in December 1994, and all work in this area of the project ceased as a consequence.

5.1.2.4 Antibiotic Susceptibility.

Antibiotic	Mixture 1A		Antibiotic	Mixture 1A		Antibiotic	Mixture 1A	
	Aero (mm)	Anaero (mm)		Aero (mm)	Anaero (mm)		Aero (mm)	Anaero (mm)
Amoxycillin AML 2	—	—	Ampicillin AMP 2	1.0	0	Ciprofloxacin CIP 1	11.5 Hazy	1.0
Amoxycillin AML 10	5.5	1.0	Gentamicin CN 10	8.0 Hazy	0.5	Cephalexin CL 30	2.5 Hazy	—
Amoxycillin AML 25	7.5	3.0 Hazy	Penicillin G P2 (v)	1.5 Hazy	—	Nalidixic Acid NA30	4.5 V Hazy	0
Metronidazole MTZ 5	0	0	Erythromycin E 10	0	1.0	Ciprofloxacin CIP 5	5.0 S Hazy	10.0
Metronidazole MTZ 50	0	0	Trimethoprim W 1.25	2.0 Hazy	0.5	Oxytetracycline OT 30	0	3.0
Cloxacillin OB 5	0	—	Cephadrine CE 30	2.0 Hazy	3.0 Hazy	Sulpha- methoxazole RL25	0	—
Chloramphenical C 10	4.5 Hazy	4.0	Cefuroxime CXM 30	8.0 Hazy	5.0 Hazy	Amikacin AK 10	5.5	1.0
Neomycin N 30	2.5 S. Hazy	7.0 Hazy	Amoxycillin Clavulanic Acid AMC 30	6.5 Hazy	1.0	Aztreonam AM 30	0	0

Table 5.1.2.4.b Results of Antimicrobial Susceptibility Test of Microorganisms Cultured in Brain Heart Infusion Broth.

The results of the antimicrobial susceptibility test for the microorganisms which had been cultured in Cooked Meat broth are presented in table 5.1.2.4.a. Unfortunately a problem was encountered when assessing the susceptibility of the organisms which had been cultured in Brain Heart Infusion broth. In all but one of the test cultures (Mixture 1A) assayed the antimicrobial susceptibility discs had moved across the agar. It was planned to repeat this experiment but unfortunately Dr Soper died before this could be done.

5.1.3 Electron Microscopy

5.1.3.1 Scanning Electron Microscopy (SEM)

5.1.3.1.1 Rat Small Bowel

The intestinal epithelium of the unulcerated areas of all the small bowel tissue, both from the control and experimental animals, had sloughed off the villi in an intact sheet (see photo. 5.1.2). This was due to autolysis which occurs rapidly in the gut once the blood supply is cut off from the cells of the gastrointestinal tract. It was possible to see, however, that in the small bowel tissue from the control animals (see photo. 5.1.2) and the animals given nabumetone that the epithelial cells, amongst intact layers of epithelium, were very close together with no visible gaps. The intestinal epithelial cells from animals which had been given an acute dose of indomethacin ID₇₅, however, did have visible gaps between them. The intestinal epithelium immediately surrounding the ulcer had remained attached to the underlying surface of the small bowel, probably because it was anchored in place by the ulcer which had penetrated into the muscle layer.

5.1.3.1.1.1 Control Rat Small Bowel Tissue

SEM of the rat duodenum, jejunum and ileum in control animals showed that very few bacteria were present in the small bowel of normal animals and those seen tended to be bacilli (0.5 µm × 2.0 µm). These bacteria looked very similar to *Escherichia coli*.

5.1.3.1.1.2 Experimental Rat Small Bowel Tissue

5.1.3.1.1.2.1 Indomethacin ID₇₅ (7.5 mg/kg) Acute-Induced Ulcers

The discrete ulcers were very focal and the dividing line between the ulcerated and normal tissue was very marked. The deepest ulcers were found to be full of bacteria, in the exponential phase of growth, and it was impossible to see any of the original villus structure under the mass of bacteria (see photo. 5.1.3). In the shallower ulcers it was possible to see truncated villi, at the ulcer edge, which appeared to be full of bacteria that had apparently burrowed into them (see photo. 5.1.3). There were also villi present at the ulcer edge which although they still retained their original structure also appeared to be full of bacteria, burrowing inside them. These 'burrowing bacteria' all seemed to be bacilli. Surrounding areas of the small bowel, which did not have ulcers,

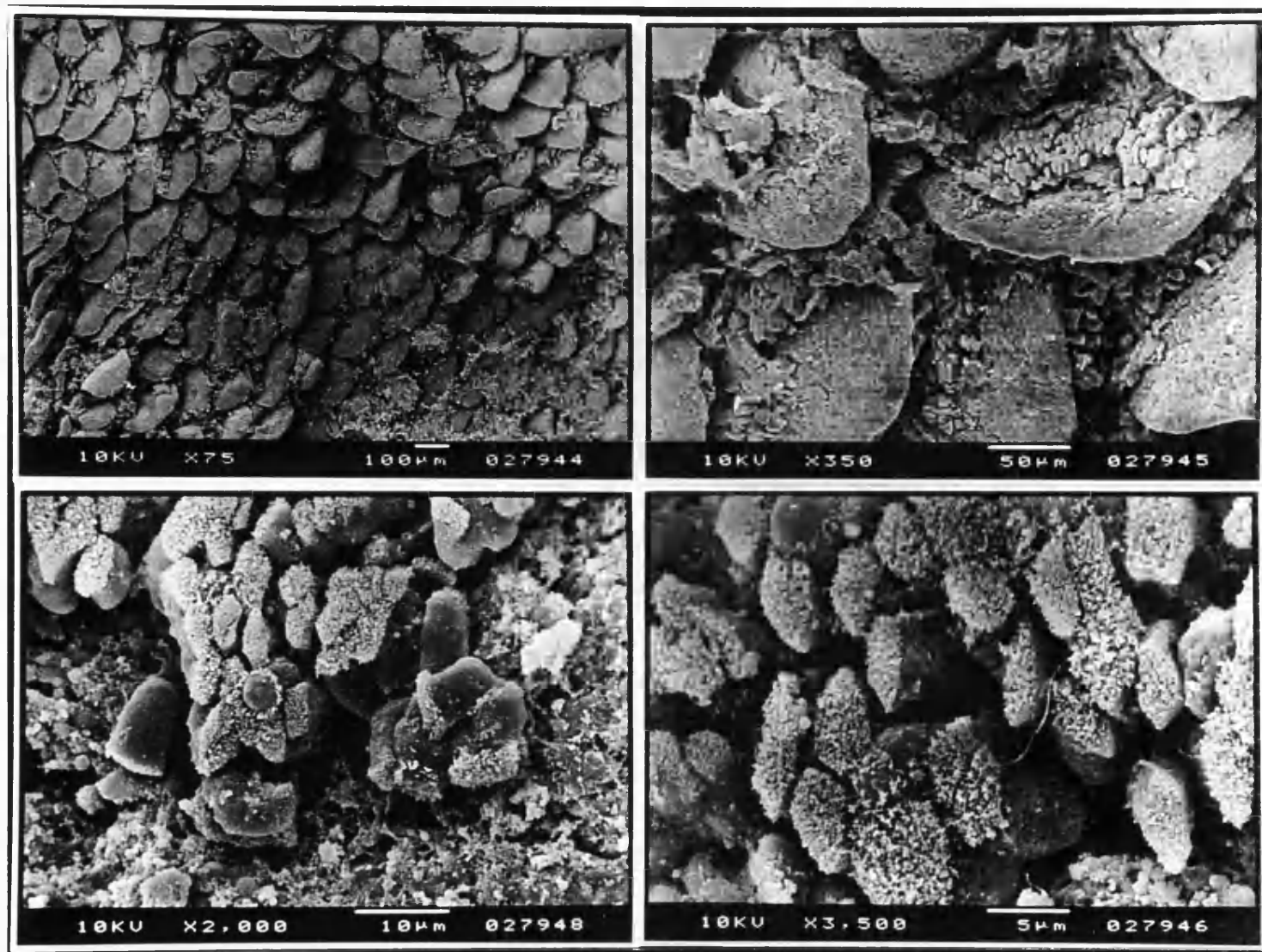


Photo. 5.1.2 Control Rat Small Bowel Tissue

had very few bacteria and was similar in appearance to that seen in control animals. There were a few ulcers that appeared to be full of just one type of bacteria and these were filamentous bacillary forms.

Bacteria seen in the small bowel ulcers of animals dosed with indomethacin included small cocci which were 1.0 μm in diameter, bacilli ranging in size from 0.5-1.0 $\mu\text{m} \times$ 1.5-2.0 μm to 1.0 $\mu\text{m} \times$ 6.0 μm , fusiform bacilli (0.5 $\mu\text{m} \times$ 4.0 μm to 0.5 $\mu\text{m} \times$ 8.0 μm) and filamentous bacillary forms ranging in size from 0.25 $\mu\text{m} \times$ 2.0 μm to $> 0.25 \mu\text{m} \times 3.0 \mu\text{m}$. There were also what appeared to be both coccobacilli (1.0 $\mu\text{m} \times$ 1.5 μm) and diplococci (0.75 $\mu\text{m} \times$ 2.0 μm) present in the ulcers. The bacilli and the fusiform bacilli were seen to be growing in close conjunction with one another and were intertwined whereas the cocci, which were far fewer in number, tended to be resting on top of the mass of bacilli. The bacilli were either occurring singly, in pairs or in short chains.

Some of these bacteria are probably *Proteus spp.* (straight rods, 0.4-0.8 μm in diameter \times 1.0-3.0 μm in length) and *Escherichia coli* (straight rods, 1.1-1.5 μm in diameter \times 2.0-6.0 μm in length) as these bacteria had already been isolated from ulcers which had excised concurrently with those taken for SEM, from the same animal.

Some of the rod-shaped bacteria could be *Klebsiella sp.* (straight rods, 0.3-1.0 μm in diameter \times 0.6-6.0 μm in length), a gram-negative facultative anaerobe which is found in short chains, similar to some of the bacteria seen within the ulcer base, and other researchers have isolated these microorganisms from the small bowel [Draser & Shiner, 1969; Draser *et al.*, 1969]. Similarly, the rod-shaped bacteria could be *Clostridium sp.* (straight rods, 0.3-2.0 μm in diameter \times 1.5-20.0 μm in length), a gram-positive anaerobe, as these species are known to be found in increased numbers within the small bowel of rats with indomethacin-induced ulceration [Kent *et al.*, 1969]. Many of the bacilli seen within the ulcer base were rod shaped with terminal swellings which can be indicative of *Bacteroides* [Holt *et al.*, 1994], a gram-negative anaerobe, and these bacteria are also known to be present in great numbers within the rat ileum [Draser & Shiner, 1969; Draser *et al.*, 1969; Simon & Gorbach, 1986, 1987].

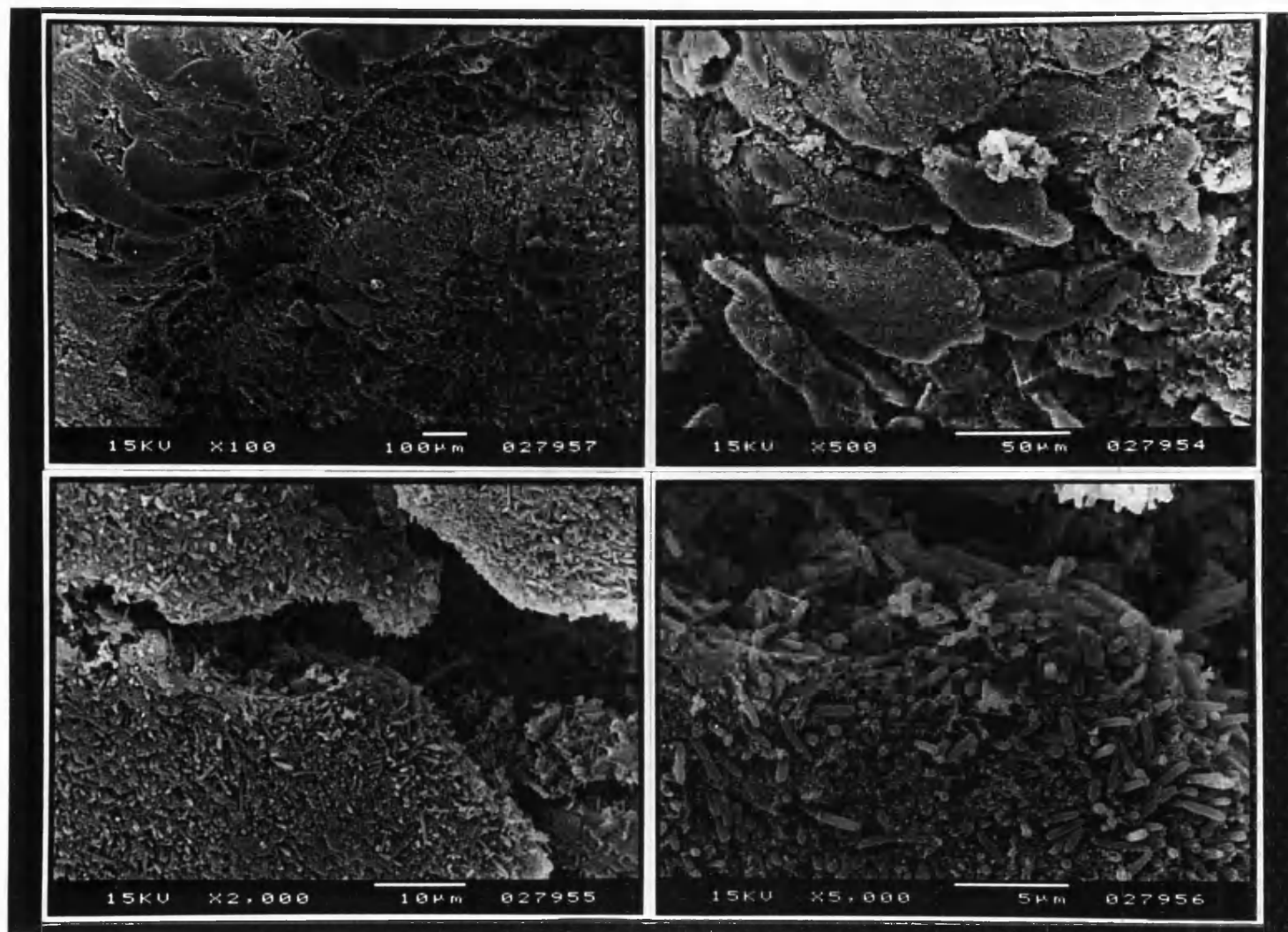


Photo. 5.1.3 Bacteria Within a Small Bowel Ulcer Taken From a Rat Dosed, Orally, Once, With Indomethacin ID₇₅ (7.5 mg/kg)

The fusiform bacilli are possibly *Fusobacterium sp.*, a gram-negative anaerobe which is a member of the family *Bacteroidaceae*, as this bacterium, slender with tapered ends [Sonnenwirth, 1980; Holt *et al.*, 1994], has also been isolated from the ileum [Draser & Shiner, 1969; Draser *et al.*, 1969].

The cocci could either be *Peptostreptococcus sp.* (0.5-1.2 μm in diameter) or *Peptococcus sp.* (0.3-1.2 μm in diameter) which are both gram-positive anaerobes or alternatively *Enterococcus sp.* (0.6-2.0 $\mu\text{m} \times 0.6-2.5 \mu\text{m}$), a gram-positive facultative anaerobe, as these are all found within the small bowel [Draser & Shiner, 1969; Draser *et al.*, 1969; Morse, 1980].

The filamentous forms are probably the same species as the segmented filamentous bacteria which have been previously reported, viewed via SEM [Garland *et al.*, 1978, 1982; Klaasen *et al.*, 1992]. These bacteria, which are found in the distal ileum of healthy adult mice [Davis & Savage, 1974; Chase & Erlandsen, 1976] and rats [Garland *et al.*, 1979], attached to Peyer's patches [Savage, 1969; Klaasen *et al.*, 1992; Jepson *et al.*, 1993], have yet to be cultured in a recognisable form [Savage, 1977; Lee, 1980; Klaasen *et al.*, 1992; Snel *et al.*, 1994] but are believed to be related to *Clostridium* [Snel *et al.*, 1994].

5.1.3.1.1.2.2 Indomethacin ID₇₅ (7.5 mg/kg) Acute Post Combined Antibiotics

There was no evidence of ulceration and only one bacterium (0.5 $\mu\text{m} \times 16.0 \mu\text{m}$) was seen out of all the samples viewed. This bacterium was possibly a strain of *Escherichia coli* which had become elongated as a result of amoxycillin [Jawetz, 1987] binding to one of the seven penicillin-binding proteins (PBP), as PBP3 is thought to be responsible for septum formation and cell division [Lambert, 1987].

5.1.3.1.1.2.3 Diclofenac ID_{112.5} (10.35 mg/kg) Chronically-Induced Ulcers

The discrete ulcers were very focal and the dividing line between the ulcerated and normal tissue was very marked. However, the diclofenac induced ulcer was surrounded by what appeared to be a ring of shrunken villi which were still relatively intact. (The equivalent of this ring in the indomethacin induced ulceration was the truncated villi full

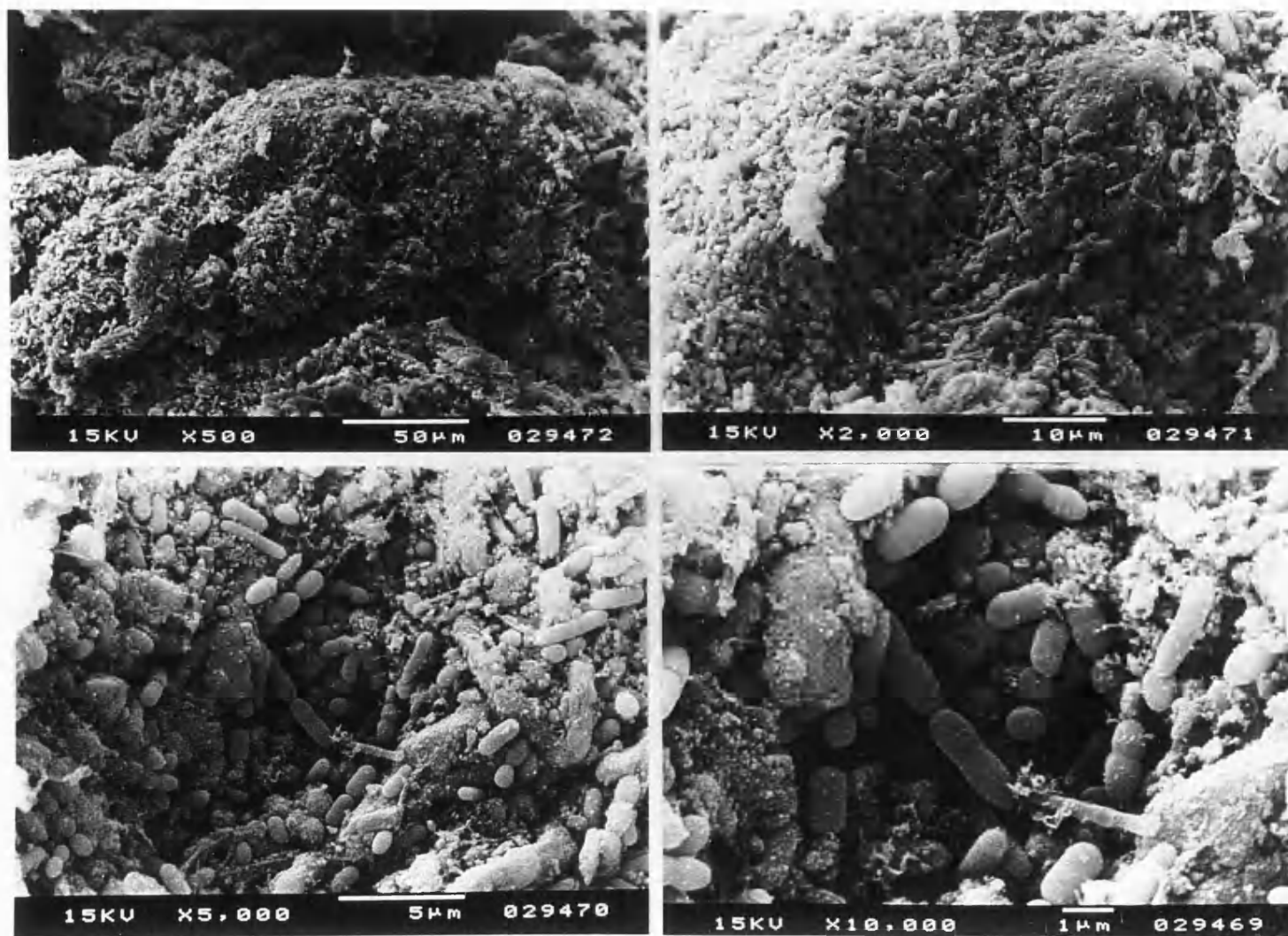


Photo. 5.1.4 Bacteria Within a Small Bowel Ulcer Taken From a Rat Dosed Orally ,Once Daily, For Four Days With Diclofenac ID₁₁₂₅ (10.35 mg/kg)

of bacteria.) The bacteria present in the ulcers were broadly the same as those seen in the indomethacin induced ulcer although there were some differences (see photo. 5.1.4). The bacilli ranged in size from $0.5\ \mu\text{m} \times 2.0\ \mu\text{m}$ to $0.75\ \mu\text{m} \times 2.5\ \mu\text{m}$ and two types of fusiform bacilli were seen ($0.5\ \mu\text{m} \times 4.0\ \mu\text{m}$, $1.0\ \mu\text{m} \times 8.0\ \mu\text{m}$). The cocci, coccobacilli and diplococci were similar to those seen in the indomethacin treated animals but no filamentous bacillary forms were visible. This is probably due to the fact that most of the diclofenac-induced ulcers occurred within the jejunum so the ulcers taken for SEM were excised from this area only and these particular bacteria only occur in the distal ileum [Davis & Savage, 1974; Chase & Erlandsen, 1976].

5.1.3.1.1.2.4 Nabumetone ID₁₂₅ (79.0 mg/kg) Acute Small Bowel Tissue

Although there were no ulcers in the small bowel tissue taken from animals treated with nabumetone a few bacteria were seen. These were present either singly or in isolated clumps of three to four types of microorganism. The bacteria present were coccobacilli ($0.5\ \mu\text{m} \times 1.0\ \mu\text{m}$ to $1.0\ \mu\text{m} \times 2.0\ \mu\text{m}$), bacilli ($0.5\ \mu\text{m} \times 1.5\ \mu\text{m}$ to $0.5\ \mu\text{m} \times 2.0\ \mu\text{m}$), fusiform bacilli ($0.75\ \mu\text{m} \times 5.5\ \mu\text{m}$) and filamentous bacillary forms ($0.25\ \mu\text{m} \times >4.0\ \mu\text{m}$).

5.1.3.1.2 Human Small Bowel

The samples taken from the human duodenum, via endoscopy, were normal in appearance in all cases except for the sample taken from an elderly man suffering with rheumatoid arthritis. The villi were all rounded with smooth tops which were made up of a covering layer of cells whose exposed surfaces were either pentagonal or hexagonal in shape, joined together in a manner similar to that seen in the chemical structure of C₆₀ ('Bucky Ball'). The cell junctions were visible in some of the samples, but in others the junctions were so close they could not be visualised. However, in the elderly man with rheumatoid arthritis the villi were very rough in appearance (like a cauliflower) with sizeable gaps between the surface cells. Within these wide cell junctions it was possible to see many bacteria which appeared to be filamentous bacillary forms (see photo. 5.1.5).

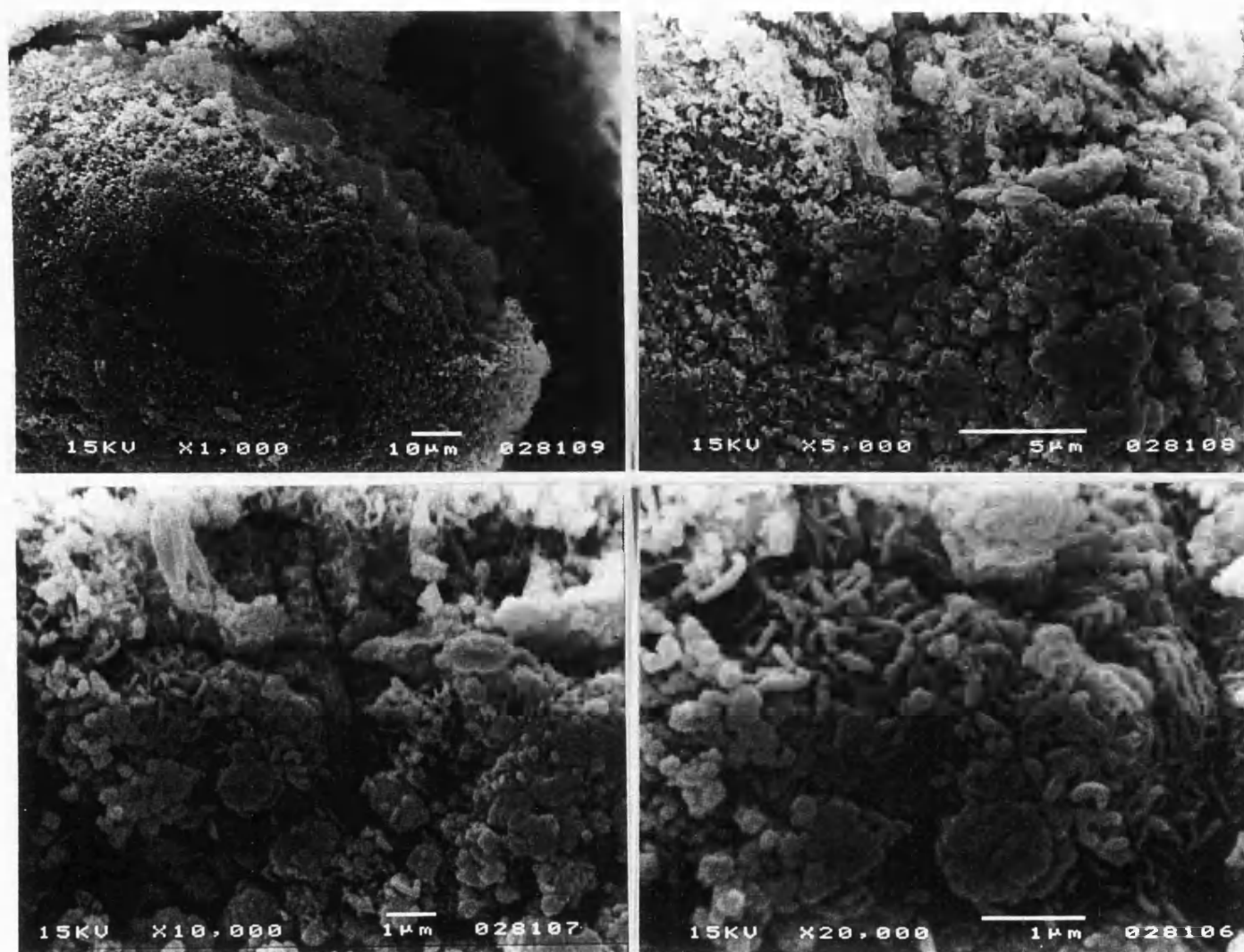


Photo. 5.1.5 Bacteria Within a Villus Tip of Human Duodenal Epithelium Taken From RA Patient on Long Term NSAID Therapy

5.1.3.2. Transmission Electron Microscopy (TEM)

5.1.3.2.1 Rat Small Bowel

5.1.3.2.1.1 Control Small Bowel Tissue

5.1.3.2.1.1.1 Distilled Water or 10% Tween 80

The clarity of the cristae within the mitochondria varied depending upon which cell the mitochondria were located and in some cells the mitochondria were very indistinct. This has been reported as a common problem when studying mitochondria in cells of the gastrointestinal tract via TEM, particularly those within Paneth cells [Madara & Trier, 1987]. The mitochondria within the cells comprising the villus were the ones which were the most distinct (see photo. 5.1.6a). Within some cells there was also a difference in the number of cristae visible within some of the mitochondria and these half empty mitochondria were initially thought to be indicative of damage, possibly caused during processing.

It has been reported, however, that mitochondria alter their internal conformation depending on whether or not they are in the resting (orthodox) or active (condensed) state. In the orthodox state the inner membrane displays the characteristic crests and the matrix, which is granular in appearance, almost fills the entire volume of the mitochondrion. This orthodox state is induced when the level of external ADP becomes low resulting in a lack of ADP available for phosphorylation. In the presence of ADP, however, on initiation of electron transport; the matrix shrinks to a volume that is only about 50 per cent of that seen in the orthodox state and the inner membrane and cristae become more tightly folded and more contorted as the respiring mitochondrion generates ATP at a maximum rate. In this condensed state, as the inner compartment of the mitochondrion contracts, fluid accumulates in the outer compartment. [Lehninger, 1975, 1982; De Robertis & De Robertis, 1980]. Thus the empty looking mitochondria observed in the control tissues are probably normal mitochondria in the active state. Ultrathin sections of tissue from animals dosed with 10% Tween 80 were very similar in appearance to animals which had been dosed with distilled water.

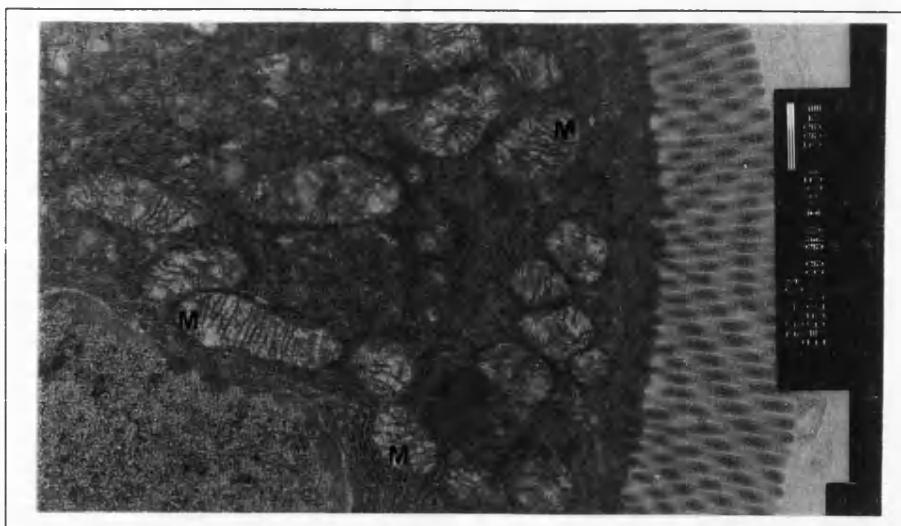


Photo. 5.1.6a



Photo. 5.1.6b

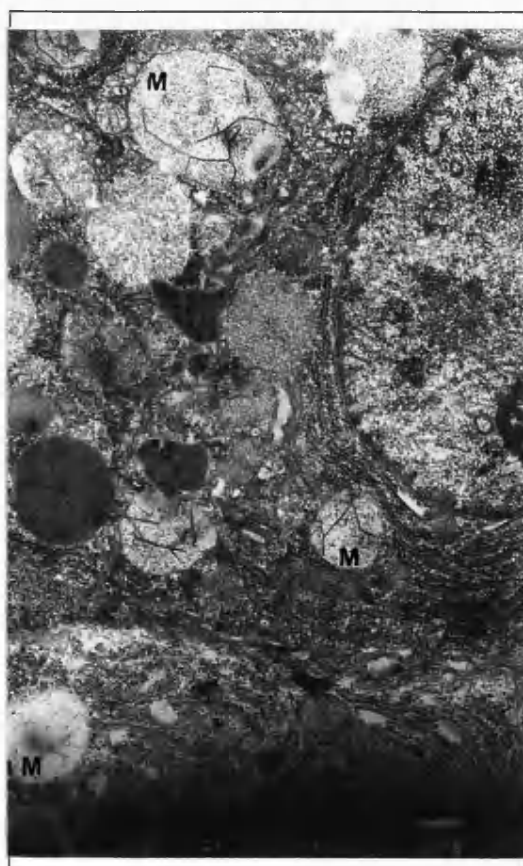


Photo. 5.1.6c

Photo. 5.1.6 Mitochondria (M) Within Epithelial Cells of Rat Small Bowel Tissue

- a. Normal Mitochondria Within Epithelial Cell of Villus Tip From Control Rat Small Bowel Tissue
- b. Damaged Mitochondria Within Epithelial Cell Adjacent to a Small Bowel Ulcer Taken From a Rat Dosed Orally, Once, With Indomethacin ID₆₀ (6.0 mg/kg)
- c. Abnormal Mitochondria Within Epithelial Cell of an Unulcerated Region of Small Bowel Tissue (Between Ulcers) Taken From a Rat Dosed Orally, Once, With Indomethacin ID₆₀ (6.0 mg/kg)

5.1.3.2.1.2 Experimental Small Bowel Tissue

5.1.3.2.1.2.1 Indomethacin ID₆₀

The mitochondria seen within the cells immediately surrounding the ulcer contained fewer cristae and did not have a healthy appearance (see photo. 5.1.6b). These mitochondria looked different to those seen in the normal control tissue which were believed to be actively respiring. In the unulcerated areas of the small bowel tissue, most of the mitochondria were similar in appearance to those seen in the normal control tissues. However, in some cells within the unulcerated areas of small bowel tissue there were a few mitochondria which did look unusual and seemed to contain disrupted cristae (see photo. 5.1.6c).

5.1.3.2.1.2.2 Tissue from Animals Dosed with Remaining NSAIDs

There appeared to be no difference between the ultrastructure of the small bowel tissue of animals dosed with the ID₆₀ values of diclofenac, nabumetone, naproxen and piroxicam when they were compared with the control tissues.

5.2. DISCUSSION - NSAIDS AND ULCER FORMATION

5.2.1 Production of Small Bowel Ulcers

5.2.1.1 Location and Distribution of NSAID-Induced Ulceration

5.2.1.1.1 Association of Ulceration With Mesentery

The NSAID-induced ulceration within the small bowel occurred predominantly within the region bounded by the proximal jejunum and the mid to distal ileum. A few of the animals (3/19) given single low doses of indomethacin, which did not develop ulcers, had erosions only in the outer regions of this area of the small bowel. The animals given a single low dose of indomethacin in which ulcers did occur tended to have them in the mid jejunum. The ulceration was on the mesenteric side and followed the blood supply so it would appear that proximity to the blood vessels within the mesentery is necessary for the ulcerative process, and the mesentery of the animals with ulcers within the small bowel was full of inflammatory cells. The duodenum is a region of the small bowel where there is no mesentery so this may explain why NSAID-induced ulceration in the rat is not found in the duodenum.

5.2.1.1.2 Association of Ulceration With pH

The pH of the small bowel contents is 7.0 all the way through to the distal ileum where it begins to rise. Raising the environmental pH from 7.0 to 7.4 can lead (depending on the pKa value) to a decrease in the proportion of un-ionised NSAID by a factor of four [Day *et al.*, 1990] and it is only the un-ionised portion of the drug that is able to pass through the lipid bilayer of the intestinal epithelial cell wall. Calculations using these raised pH figures in the Henderson-Hasselbalch equation for all the NSAIDs investigated indicate that when the unconjugated drugs are predominantly in the ionised form the majority of them are retained within the terminal ileum. Thus very little absorption by the small bowel, of the unconjugated drug, goes on in this area as being ionised the NSAIDs are trapped within the terminal ileum; while they pass through to the caecum, and possibly this is the reason why very little ulceration if any occurs in this region.

5.2.1.1.3 Association of Ulceration With Peyer's Patches

The lower acute doses of indomethacin resulted in small discrete ulcers, within the jejunum and ileum, which occurred at regular intervals, approximately one centimetre apart. As the ulceration increased in severity, in response to an increase in drug concentration, the discrete ulcers occurred approximately every 0.5 centimetres. A similar result was found in the small bowel of rats dosed chronically with diclofenac. These discrete full thickness ulcers were associated with Peyer's patches and the area of necrosis involved the lymphoid follicle itself and the adjacent tissue. There were also erosions immediately above Peyer's patches in other regions the small bowel taken from animals exhibiting ulceration. Other researchers have reported that scattered discrete indomethacin-induced ulcers occur predominantly over the Peyer's patches within the small bowel [Stewart *et al.*, 1980; Del Soldato *et al.*, 1987], although another group investigating the formation of indomethacin-induced ulceration, solely within the jejunum, did not find this [Anthony *et al.*, 1993]. These conflicting results could be a consequence of ulcers forming via alternative mechanisms, or by similar mechanisms but with certain steps being of greater or lesser importance, depending on their position within the small bowel. In some of the ulcerated small bowel tissue, viewed via light microscopy, there was evidence of microscopic erosions in areas away from the fully developed ulcers associated with the Peyer's patches. It is possible that damage caused by indomethacin to cells of the intestinal epithelium away from Peyer's patches does not initiate the catastrophic inflammatory response that may occur if the cell damage happens in close proximity to a Peyer's patch.

Aggregates of mucosal lymphoid follicles can be found throughout the small bowel, but the largest and most abundant occur within the ileum where they are called Peyer's patches [Madara & Trier, 1987]. Peyer's patches are a known site of bacterial invasion by many microorganism, *Shigella flexneri* [Sonnenwirth, 1980], *Yersinia enterocolitica* [Grutzkau *et al.*, 1990; O'Loughlin, 1995] and *Vibrio cholerae* [Kuby, 1997] in man and *Salmonella spp.* in both man [Kuby, 1997] and the rat [Sonnenwirth, 1980]; although in rats this is believed to be inhibited in the distal ileum where the Peyer's patches are colonised by segmented filamentous bacteria [Garland *et al.*, 1979; Savage, 1979]. Peyer's patches, which extend down to the submucosa, are responsible for the initiation

and expression of mucosal immunity [Bockman & Cooper, 1973; Owen & Jones, 1974; Owen, 1977] and are thought to be the main site of antigen handling by the intestine [Kagnoff 1974, 1987a].

The epithelium covering the rounded surface of these follicles is made up of absorptive cells, goblet cells and specialised epithelial cells called M cells [Owen & Jones, 1974; Owen & Nemanic, 1978]. These M cells only occur in the region of lymphoid follicles and are associated with sites of multiple mononuclear cell infiltration as migrating lymphocytes, macrophages and plasma cells are visible in the intercellular space between M cells and neighbouring epithelial cells. Evidence suggests that M cells transport intraluminal antigens to the lymphoid tissue of Peyer's patches [Bockman & Cooper, 1973; Owen, 1977; Wolf *et al.*, 1981] and many of these cells have central hollows, containing mononuclear cells, which may facilitate this [Madara & Trier, 1987]. The mononuclear cells which are on the epithelial side of the basal lamina are able to pass easily back across the basal lamina into the lymphoid follicles. In addition to mononuclear cells, B and T lymphocytes also congregate within the central hollow of M cells [Bhalla & Owen, 1983]. The M cells therefore appear to transport microorganisms and macromolecular antigens from the intestinal lumen to the lymphoid follicles where they can be carried to mesenteric nodes and into the systemic circulation [Cebra *et al.*, 1977; Walker & Isselbacher, 1977]. Thus this is also a site to which neutrophils can readily migrate in response to chemotactic cytokines released by activated macrophages and other mononuclear cells which have been in contact with bacteria and/or their toxins.

The formation of ulcers above Peyer's patches may therefore be due to an inflammatory response initiated by indomethacin-induced damage to epithelial cells in close proximity to them. Peyer's patches appear to be a site of attraction to various microorganisms [Garland *et al.*, 1979; Savage, 1979; Sonnenwirth, 1980; Wolf, 1988; O'Loughlin, 1995; Sansonetti *et al.*, 1996; Kubly, 1997] where they bind preferentially to M cells [Savage, 1969; Klaasen *et al.*, 1992; Jepson *et al.*, 1993; Sansonetti *et al.*, 1996] and this in itself can lead to ulcer formation [Wassef *et al.*, 1989; Jepson *et al.*, 1993]. Small clusters of bacteria (3-4 types), which may have been associated with Peyer's patches, were visible in some regions of the small bowel tissue from nabumetone treated animals, viewed via

SEM. This was in contrast to the rest of the small bowel tissue where bacteria were extremely rare. Thus when the initial microscopic erosions, which form in the intestinal epithelium as a result of indomethacin, occur in the region of a Peyer's patch there may already be a number of bacteria present enabling bacterial toxins to readily infiltrate this area of compromised mucosa. This initiates an inflammatory response which results in neutrophils rapidly migrating to this area. Once the inflammatory cascade has started the surrounding tissue is damaged by neutrophil degranulation as the region is swamped with reactive oxygen metabolites and proteases, causing necrosis to the surrounding tissue.

In areas away from the Peyer's patches, however, the initial indomethacin-induced damage does not elicit an inflammatory response immediately as there are few if any bacteria in the immediate vicinity. In the absence of luminal aggressors this type of damage, where the basal lamina remains intact, would usually be repaired by an increase in intestinal epithelial cell turnover [Eastwood, 1977], however, cellular turnover is decreased after NSAID ingestion due to inhibition of prostaglandin synthesis [Robert, 1976, 1979]. (Damage to the gastric epithelium is repaired via restitution, a process whereby surrounding epithelial cells rapidly migrate over the defect, re-establishing epithelial continuity [Ito & Lacy, 1986]. Restitution, which does not involve cell division, is not attenuated by NSAIDs and does not require prostaglandins [Levi & Shaw-Smith, 1994]. Possibly a similar mechanism occurs within the small bowel.) Once the bacterial overgrowth has started in the region of the Peyer's patch, however, as the microorganisms begin to multiply rapidly in the presence of metabolites supplied by the necrotic tissue, bacterial toxins begin to diffuse and eventually invade these areas of microscopic erosion further away. This then initiates an inflammatory response causing ulcers to form away from Peyer's patches. In addition, the increase in bacterial numbers in the vicinity of the Peyer's patch probably overwhelms the hosts immunological defence mechanisms enabling microorganisms to invade the lymphoid-follicle itself resulting in the full thickness ulcer.

5.2.1.1.4 Effect of NSAID-Induced Ulceration on Small Bowel Length

As the severity of ulceration increased there was a corresponding shortening of the small bowel. This was most significant in the rats which had the longitudinal ulcers and was

accompanied by the greatest weight loss. Similar findings have been reported by another group [Matsumoto *et al.*, 1994]. There was a significant correlation between weight and small bowel length in both control animals and animals which had been dosed chronically with NSAIDs but had not developed ulcers. There was no correlation between weight and small bowel length, however, in animals which had been given a single dose of an NSAID and also had not developed ulcers. This was probably due to the fact that a single dose of the newer NSAIDs had very little effect on the small bowel, whereas a single dose of the older NSAIDs did. All the NSAIDs had some effect, however, when given chronically and thus it would seem to indicate that NSAIDs have an effect on small bowel length even in the absence of ulceration.

One possibility is that, in spite of cyclooxygenase inhibition by NSAIDs, there may still be an increase in prostaglandins, some of which are vasoconstrictors. Long term use of NSAIDs, such as indomethacin, can result in diaphragm-like small bowel strictures [Bjarnason *et al.*, 1988a; Levi *et al.*, 1990]. These may occur as multiple, thin, concentric diaphragm-like septae (arising from submucosal fibrosis) [Lang *et al.*, 1988], which cause a narrowing of the lumen [Levi & Shaw-Smith, 1994]. Perhaps the decrease in small bowel length observed in the rat after NSAID administration may also be due to some form of fibrosis.

5.2.1.1.5 Association of Ulceration With Increasing Concentration of NSAID

5.2.1.2.5.1 Gastric Ulceration

Indomethacin-induced ulceration was only found within the stomach on two occasions and this was when the second batch of Interfauna rats were given an acute dose of indomethacin ID₁₂₅. In one group (n = 3) which had been starved 21 hours before and 3 hours after the acute dose of indomethacin ID₁₂₅, two animals were found to have stomach ulcers. These ulcers were found in the antrum and similar findings have been reported previously in animals which have been starved before and after an acute dose of indomethacin [Sato *et al.*, 1981, 1982]. This is probably a result of the antrum being exposed to the indomethacin for a longer period of time than usual, as food was withheld until three hours after dosing. In addition to this the drug concentration within the empty stomach of these animals was probably greater than that found in animals which had been

allowed free access to food. It has been reported that the degree of antral ulceration within the stomach of re-fed rats is dependent on the type of food they are given as a low-residue diet significantly decreased the ulceration whereas the reverse was found when rats were re-fed with high-bulk substances [Sato *et al.*, 1982]. This is thought to be due to the gastric mucosa being more susceptible to mechanical damage as the prolonged exposure to indomethacin would have resulted in a depletion of cytoprotective prostaglandins. Similarly, high-bulk foodstuffs were found to increase ulcer formation within the small bowel of re-fed rats [Sato *et al.*, 1982]. The other group (n = 3) of Interfauna rats, that were given an acute dose of indomethacin ID₁₂₅, were allowed free access to food before and after dosing and one of these animals developed ulcers within the antrum. This may have been due to the fact that the animal did not eat any food before and after dosing even though it was freely available or alternatively this batch of Interfauna rats were genetically more susceptible to gastric ulceration.

5.2.1.2.5.2 Small Bowel Ulceration

The difference between concentrations of what was and what was not an ulcerogenic dose of NSAID in the case of indomethacin was small. In both acute and chronic instances raising the dose of indomethacin slightly resulted in ulceration which was out of proportion to the increase in the dose. Indomethacin ID₅₀ (5.0 mg/kg) acute caused very little ulceration, if any, in Wistar rats whereas ID₆₀ (6.0 mg/kg) acute could result in quite severe ulceration. Indomethacin ID₄₀, (4.0 mg/kg) when given to animals for three consecutive days, caused very little ulceration whereas indomethacin ID₄₅ (4.5 mg/kg) induced extremely severe ulceration after just two doses.

Similarly diclofenac ID₁₀₀ (9.2 mg/kg) administered daily for seven days caused very little ulceration whereas diclofenac ID_{112.5} (10.35 mg/kg) administered for four days was life threatening and the experiment had to be terminated early. Thus, once the concentration of these two drugs passes the ulcerogenic threshold the degree of ulceration increases dramatically. This threshold is due to the length of time that the drug takes to reach a steady state within the plasma. The higher doses of indomethacin reach this level very quickly whereas the smaller doses need to be administered over a longer period of time in order to reach this stage. Once the drug has reached a steady state within the plasma,

biliary excretion of these two NSAIDs (indomethacin and diclofenac), which both undergo enterohepatic recirculation in the rat [Hucker *et al.*, 1966; Yesair *et al.*, 1970; Duggan *et al.*, 1975; Riess *et al.*, 1975], results in the small bowel being continuously exposed to drug over a significant period of time. The time at which a steady state is achieved within the plasma can vary considerably between individuals (human and animal) which is why it is difficult to produce a dose response curve with these compounds [Orme, 1985; Grennan *et al.*, 1985].

The other NSAIDs used in this study, fenbufen, nabumetone, naproxen and piroxicam, did not cause any form of ulceration in the animals even when the ID₁₂₅ dose was administered daily over the course of a month. The differences in ulcerogenesis between the NSAIDs used during this project may be due in part to their pharmacokinetic properties.

5.2.3 Pharmacokinetics of the NSAIDs Used

5.2.3.1 Metabolism

All six NSAIDs used in these experiments are lipid soluble and readily absorbed from the gastrointestinal tract. Metabolism (biotransformation) of foreign compounds (xenobiotics) occurs primarily in the liver [Boulton *et al.*, 1996] and consequently this is the site where the majority of drugs, NSAIDs included, are metabolised. These metabolic processes usually cause an increase in water solubility (frequently accompanied by a decrease in lipid solubility) which enable compounds to be more readily eliminated renally [Wingard *et al.*, 1991]. Phase I reactions (oxidation, reduction, hydrolysis) usually convert the drug to a more polar metabolite by introducing a functional group (-OH, -NH₂, -SH) and if these metabolites are sufficiently polar they are readily excreted. However, if the phase I metabolite is not readily eliminated it can undergo a phase II reaction whereby an endogenous substrate, such as glucuronic acid, sulphate, acetic acid or an amino acid, combines with an existing or newly established functional group to form a highly polar conjugate [Correia & Castagnoli, 1987]. The six NSAIDs studied undergo phase II metabolism and in man become conjugated to glucuronic acid [Brune & Lanz, 1985] (glucuronic acid is a six carbon sugar moiety with a molecular weight of 194), whereas in the rat they are usually conjugated to sulphate [Riess *et al.*, 1975;

Brune & Lanz, 1985]. The factors determining whether or not a compound is secreted into the bile and subsequently enters the small bowel, are the possession of a large polar group, which Phase II metabolites in man possess, and the overall molecular weight. This is species dependent and in man the conjugate has to have a molecular weight in excess of 500 whereas in rat it only has to be greater than 300. Thus some phase II metabolites of NSAIDs, which in man are eliminated renally, undergo biliary excretion in the rat.

Diclofenac is conjugated in the liver and, in humans, is primarily eliminated, with its conjugated metabolites, via the kidneys (70 per cent) [Riess, *et al.*, 1978; Brune & Lanz, 1985]. Diclofenac, with a molecular weight of 318, readily enters the bile in man [Riess, *et al.*, 1975, 1978] as after conjugation with glucuronic acid it exceeds the 500 molecular weight threshold. In the rat excretion is predominantly biliary [Riess, *et al.*, 1978] and in contrast to man, where all the native drug is metabolised, the bile of the rat is found to contain considerable amounts of unchanged drug as well as metabolites which can yield native drug on hydrolysis. Thus in the rat diclofenac undergoes extensive enterohepatic recirculation [Riess *et al.*, 1975] and this continued exposure may explain why small bowel ulcers form more readily in the rat than in man.

Fenbufen is a prodrug which is inactive in its native form but undergoes extensive metabolism within the liver, resulting in the formation of its active metabolite, [1.1'-biphenyl]-4-acetic acid (BPAA) together with other inactive metabolites [Chiccarelli *et al.*, 1980; Birnbaum *et al.*, 1982]. BPAA, with a molecular weight of 212, is conjugated in the liver and almost totally eliminated via the kidneys in both man and in the rat without undergoing any significant enterohepatic recirculation [Brune & Lanz, 1985]. Consequently there is little exposure to the drug in the small bowel of both man and rat and this may partly explain its lack of ulcerogenic properties.

Nabumetone, a nonacidic NSAID, is also a prodrug which is inactive in its native state and is converted, through extensive metabolism within the liver, to its active metabolite 6-methoxy-2-naphthylacetic acid (6MNA) together with other minor metabolites [Hyneck, 1992]. 6MNA, with a molecular weight of 216, is eliminated via the kidneys as

a conjugate or as a demethylated metabolite, and does not undergo significant enterohepatic recirculation in man [Brett *et al.*, 1988, 1992; Hyneck, 1992] as after conjugation with glucuronic acid it remains below the 500 molecular weight threshold. Similarly nabumetone does not undergo enterohepatic recirculation in the rat, (less than one per cent of the dose is found in the bile [Melarange *et al.*, 1992]). Consequently there is little exposure to the drug in the small bowel of both man and rat and this may partly explain its lack of ulcerogenic properties.

Naproxen is metabolised in the liver and after conjugation is almost totally eliminated via the kidneys [Segre, 1973; Brune & Lanz, 1985]. Owing to the fact that it has a molecular weight of 230, it does not enter the bile in man, as after conjugation with glucuronic acid both naproxen and its major metabolite remain below the 500 molecular weight threshold. Consequently naproxen undergoes very little enterohepatic recirculation in man whereas it probably does occur in the rat.

Piroxicam is conjugated in the liver followed by primary elimination in the urine (65 per cent) with the remainder being excreted in the faeces. Secondary peaks, approximately six hours after administration, coupled with a long half life suggests that piroxicam undergoes enterohepatic recirculation in man [Wiseman & Hobbs, 1982; Hobbs, 1983; Brune & Lanz, 1985], although this supposition has been disputed [Verbeeck *et al.*, 1986]. However, with a molecular weight of 331, piroxicam is probably incorporated into the bile as after conjugation with glucuronic acid it exceeds the 500 molecular weight threshold. Similarly piroxicam probably undergoes enterohepatic recirculation in the rat although the plasma half-life of five hours in this species is less than that found in man [Schiantarelli *et al.*, 1981].

Indomethacin is metabolised by the liver and after conjugation in man, is predominantly excreted in the urine, as are its conjugated metabolites (although a small amount is found in the faeces) [Duggan *et al.*, 1975; Brune & Lanz, 1985]. With a molecular weight of 358 indomethacin readily enters the bile in man, as conjugated with glucuronic acid it exceeds the 500 molecular weight threshold. As a consequence indomethacin undergoes extensive enterohepatic recirculation in both man [Hucker *et al.*, 1966; Duggan *et al.*,

1975; Kwan *et al.*, 1976] and the rat [Hucker *et al.*, 1966; Yesair *et al.*, 1970; Duggan *et al.*, 1975] with the result that the small bowel is re-exposed to the native drug after the conjugates have been deconjugated by the bacterial β -glucuronidase and sulphatase of the indigenous microflora [Lewis & Gorbach, 1972; Scheline, 1973; Plaa, 1975]. Thus the parent drug is available for reabsorption and the cycle is repeated. The extensive enterohepatic recirculation in the rat is believed to be the primary reason for its ulcerogenic properties since indomethacin-induced ulcers do not occur in animals which have undergone bile duct ligation [Brodie *et al.*, 1970]. Indomethacin-induced ulceration, of equal severity, also occurs when indomethacin is administered parenterally, either subcutaneously [Brodie *et al.*, 1970] or intravenously [Yesair *et al.*, 1970]. Animals with Thiry loops, however, when they are given indomethacin parenterally, do not develop ulcers within the Thiry loops, although ulceration does occur in the rest of the small bowel [Brodie *et al.*, 1970]. This confirms that the gastrointestinal mucosa has to be in contact with the drug in order for the ulcerative process to begin and with parenteral dosing this can only occur via enterohepatic recirculation. Similarly, animals which are starved for 24 hours after an oral dose of indomethacin are protected from indomethacin-induced ulceration because withholding food inhibits enterohepatic circulation [Brodie *et al.*, 1970]. However, when the rat is starved prior to an oral dose of indomethacin, and the food is returned shortly afterwards, the ulceration is more severe because enterohepatic circulation has been enhanced as a result of the hungry rat's increased food intake.

5.2.3.2 Effect of pKa Values

All the NSAIDs used during this project are weak acids except for nabumetone. In regions of low pH, as in the stomach, they are predominantly in a un-ionised form and as such are able to pass through the lipid bilayer of cells which constitute the gastric epithelium. In the instance of diclofenac, naproxen and indomethacin, which have the lowest pKa values, the drugs become ionised once inside the intracellular compartment, where the pH is maintained around 7.4, and as a result become trapped within the cells. This phenomenon of 'ion-trapping' does not occur significantly with the other three NSAIDs used during this study as they are predominantly in a un-ionised state within the cells of the gastric epithelium and are thus able to pass back through the lipid bilayer into

the gastric lumen. Fenbufen and piroxicam, because they have a higher pKa value and remain almost completely un-ionised until they reach a pH of approximately two pH units above their respective pKa values, and nabumetone because it is a neutral compound unable to dissociate.

To a lesser extent the process of 'ion trapping' continues within the small bowel. Although at pH 7.0 a predominant portion of the three drugs, diclofenac, naproxen and indomethacin are ionised, a small proportion is still un-ionised and it is this portion which is able to pass through into the epithelial cells of the small bowel and become trapped as a result. With naproxen this is only able to occur when the drug initially passes down the gastrointestinal tract, as once absorbed and metabolised by the liver it is excreted in the urine via the kidneys. However, in the case of indomethacin and diclofenac, which both undergo efficient enterohepatic recirculation in the rat [Hucker *et al.*, 1966; Yesair *et al.*, 1970; Duggan *et al.*, 1975; Riess *et al.*, 1975], the small bowel is exposed repeatedly to the drug, long after the initial oral administration.

The other three NSAIDs, fenbufen, piroxicam and nabumetone, are still un-ionised as they pass through the small bowel, fenbufen and piroxicam because their higher pKa values mean that only an insignificant proportion dissociates at pH 7.0, and nabumetone because it is a neutral compound. As a result these three compounds are still actively absorbed as they pass down through the small bowel and this is in fact their main site of absorption. As with the gastric epithelial cells these drugs remain un-ionised within the cells of the intestinal epithelium so damage due to 'ion trapping' cannot occur. Piroxicam, like diclofenac and indomethacin, undergoes enterohepatic recirculation, but continual exposure to the small bowel, after the initial administration, does not cause significant damage due to its higher pKa value. Fenbufen and nabumetone are prodrugs and as such have very little activity in their native form and only acquire antiinflammatory properties after they have been metabolised by the liver into the active metabolites. Thus the small bowel is only repeatedly exposed to a single dose of diclofenac, indomethacin and piroxicam.

5.2.3.2.1 Comparison Between Diclofenac and Indomethacin

It would be expected from the pKa values that diclofenac, which has a lower pKa value than indomethacin, would be at least as ulcerogenic in rats, as these two drugs both undergo enterohepatic recirculation in the rat [Hucker *et al.*, 1966; Yesair *et al.*, 1970; Duggan *et al.*, 1975; Riess *et al.*, 1975]. In some ways indomethacin and diclofenac are very similar as both have structural formulae which contain two aromatic rings that are twisted relative to one another [Sallman, 1979]. However, despite their similarities there are important differences in their chemical properties. Diclofenac has been reported to enhance the sequestration of arachidonic acid into lipid (largely triglyceride) pools [Day *et al.*, 1990] from where it can be re-incorporated into the inner membrane of the cell. This property is most marked in monocytes and is accompanied by a decrease in the production of leukotrienes and other products of the enzyme 5-lipoxygenase [Ku *et al.*, 1985].

All NSAIDs are inhibitors of cyclooxygenase (COX-1 and COX-2), indomethacin being the most potent inhibitor, and it has been proposed that the inhibition of this enzyme causes all the arachidonic acid, which has been cleaved from the membrane by the enzyme phospholipase A₂, to be channelled into the production of leukotrienes via the enzyme 5-lipoxygenase [Rainsford, 1987; Bjarnason *et al.*, 1989]. Leukotrienes are very inflammatory compounds which are believed to be responsible for some of the NSAID-induced damage to the gastrointestinal tract. The fact that diclofenac prevents some of the surplus arachidonic acid, which is a direct result of the inhibition of cyclooxygenase, from being channelled into increased leukotriene synthesis [Ku *et al.*, 1985], is probably why it is less ulcerogenic than indomethacin.

5.2.4 Effect of Indomethacin on Small Bowel Permeability

The results obtained via scanning electron microscopy (SEM) and transmission electron microscopy (TEM) appeared to reveal some of the initial steps of indomethacin-induced ulcerogenesis. The epithelial cells covering the villi of human duodenum samples taken from patients who were not taking NSAIDs, viewed via SEM, were very close together and the cell junctions were difficult to discern. In patients taking NSAIDs, however, the cell junctions were clearly visible and in one elderly man who was suffering with

rheumatoid arthritis there were sizeable gaps between the epithelial cells. Similar gaps were found in the small bowel tissue taken from rats dosed with indomethacin ID₇₅. The samples of unulcerated rat small bowel, which had also been taken from animals dosed with indomethacin, appeared to show damaged mitochondria within some of the cells of the intestinal epithelium, when viewed via TEM. These were different in appearance to the damaged mitochondria seen within cells immediately surrounding an ulcer.

It has been reported that within six hours of administering a dose of indomethacin to humans there is an increase in small bowel permeability [Bjarnason *et al.*, 1992a]. This initial response is likely to be due to a direct topical effect of the drug and is probably a result of the drug being trapped within the cells of the intestinal epithelium. NSAIDs are capable of uncoupling oxidative phosphorylation within the mitochondria of cells of the intestinal epithelium [Brody, 1956; Penniall, 1958; Packer *et al.*, 1959; Jeffrey & Smith, 1959; Whitehouse, 1964; Smith & Dawkins, 1971; Kawai *et al.*, 1985], and it has been reported that the NSAIDs with the lower pKa values are more effective uncouplers [Mahmud *et al.*, 1996]. Experiments conducted *in vitro*, with rat liver mitochondria, show that there is a significant inverse correlation between the NSAID concentration required for maximum stimulation and the pKa value [Mahmud *et al.*, 1996]. These findings are in agreement with the knowledge that some acidic aromatic compounds, such as 2,4-dinitrophenol, are able to disrupt the tight coupling of electron transport and phosphorylation by carrying protons across the inner mitochondrial membrane thus preventing phosphorylation of ADP to ATP [Lehninger, 1975; Stryer, 1981], and NSAIDs are also acidic aromatic compounds. In the presence of such uncouplers, electron transport from NADH to O₂ proceeds normally, but ATP is not formed by the mitochondrial ATPase as the proton-motive force across the inner mitochondrial membrane is dissipated [Stryer, 1981]. Indomethacin is particularly toxic to cells, as not only does it uncouple oxidative phosphorylation, it also inhibits the respiratory chain by preventing phosphorylation, via suppression of respiration, at multiple sites [Tokumitsu *et al.*, 1977]. ATP is necessary for the maintenance of the tight junctions within the intestinal epithelium [Meza *et al.*, 1980; Madara & Dharmasathaphorn, 1985; Madara *et al.*, 1986, 1987] so a direct result of a cessation of ATP production would be for these tight junctions to break down, leading to a widening within the intercellular space

between the cells, thus allowing noxious and toxic substances to enter from the lumen into the compromised intestinal epithelium.

Additional initial damage within the cell could be due to the NSAID inhibiting various enzymes involved in glycolysis and/or the tricarboxylic acid cycle. NSAIDs have been shown to inhibit dehydrogenases [Smith & Dawkins, 1971] and such enzymes play an important role in the tricarboxylic acid cycle which also occurs within the mitochondria. Inhibition of these enzymes would prevent the formation of NADH and FADH₂, important products of the tricarboxylic acid cycle, resulting in a depletion of these substrates which are required for the electron transport chain, itself necessary for oxidative phosphorylation [Lehninger, 1975; Stryer, 1981]; thus leading to a reduction in ATP. Both occurrences could explain the changes seen in the mitochondria of cells within the small bowel tissue, viewed via transmission electron microscopy, of rats dosed with indomethacin; which has also been reported by other researchers [Bjarnason *et al.*, 1993; Bjarnason & Hayllar, 1996]; and the widening of the intercellular spaces in the intestinal epithelium seen via scanning electron microscopy.

Ultimately the uncoupling of oxidative phosphorylation and the inhibition of the tricarboxylic acid cycle could lead to cell death. The cytoplasm of all cells contains many ions and charged molecules as does the extracellular space. ATP is required for the transfer of many molecules across membranes in order to maintain the ion balance both within the intracellular compartment and between it and the extracellular space [Harrison & Lunt, 1980]. Failure to maintain this ion balance can lead to membrane disruption as the cell swells and bursts resulting in the intracellular contents being exposed to the outside milieu. Such necrosis is cytotoxic to surrounding cells in the intestinal epithelium as many of the cell contents are chemotactic. Recent studies have shown that mammalian mitochondria produce N-formylmethionyl peptides, similar to the protein synthesis-initiation sequence in bacteria, which are potent chemoattractants for neutrophils [Carp, 1987]. Thus the release of such mitochondrial contents as a result of tissue injury or anoxia could explain the recruitment of neutrophils to a non-infectious inflammatory site [Malech & Gallin, 1987]. The microscopic erosions, visible in the rat

small bowel tissue from rats dosed with indomethacin, which were away from Peyer's patches, however, did not appear to be at the centre of an inflammatory response.

5.2.5 Pretreatment With Antibiotics

5.2.5.1 Individually

The degree of ulceration produced by an acute dose indomethacin ID₆₀ was significantly reduced by pretreatment for three days with each of the antibiotics amoxycillin, metronidazole and cloxacillin as described previously. This would suggest that some of the microflora within the small bowel, which are susceptible to these antibiotics, are responsible in some way for the ulcerative process. Bacteria have been implicated as having a role in NSAID-induced ulceration [Kent *et al.*, 1969] although the identity of all the ones which are involved in ulcerative pathogenesis is unknown. Germ-free rats do not develop NSAID-induced ulcers [Robert & Asano, 1977] although they do when mono-contaminated with *Escherichia coli* [Robert & Asano, 1977]. The ulceration under these circumstances, however, is not as severe and when germ-free rats are infected with the small bowel flora of normal rats the degree of ulceration is similar in severity to that seen in conventional animals [Robert & Asano, 1977].

5.2.5.1.1 Metronidazole

The animals pretreated with metronidazole, three days prior to the acute dose of indomethacin ID₆₀, had very little ulceration although what there was occurred within the proximal/midjejunum. Therefore it would appear that the drug had killed one or some of the bacteria responsible for part of the ulcerative process and in this instance these species are mostly anaerobic although ulceration was greatly reduced in the first half of the small bowel as well when compared to control animals (see fig. 5.1.1.3.1). The bactericidal action of metronidazole is specific for obligate anaerobes [Pratt & Fekety, 1986] and *in vitro* it is effective against *Bacteroides fragilis* and other *Bacteroides spp.*, *Fusobacterium spp.*, *Clostridium perfringens*, *Clostridium difficile* and other *Clostridium spp.* [Sigeti *et al.*, 1983; Pratt & Fekety, 1986; Elliot & Stone, 1990; Church *et al.*, 1996]. It is less active against anaerobic gram-positive cocci but most strains of *Peptococcus* and *Peptostreptococcus* are susceptible [Pratt & Fekety, 1986;

Church *et al.*, 1996]. Metronidazole is able to diffuse into both aerobic and anaerobic bacteria but it is only within anaerobic bacteria that it is reduced to an unstable intermediate which rapidly causes cell death by inhibiting DNA synthesis [Edwards, 1977; Sigeti *et al.*, 1983]. It is thought to interact with biochemical pathways that are only present in obligate anaerobes [Edwards, 1977] but it has been shown to be effective against facultative anaerobes, such as *Escherichia coli* and *Proteus spp.*, under strict anaerobic conditions [Ingham *et al.*, 1980]. Metronidazole was ineffective against the three isolates and the two mixtures which were used in the antimicrobial susceptibility test which would appear to indicate that at least one of the bacteria with a role in the ulcerative process had not been isolated.

5.2.5.1.2 Cloxacillin

Animals pretreated for three days with cloxacillin had very little ulceration in the small bowel, less than the group treated with metronidazole, and similarly to the metronidazole group the ulceration occurred within the proximal/midjejunum with no ulceration at all in the latter half of the small bowel. This would seem to indicate that cloxacillin is effective against one or some of the bacteria responsible for part of the ulcerative process and in this instance these species are mostly anaerobic as ulceration within the distal region of the small bowel was reduced in comparison with the control animals (see fig. 5.1.1.3.1). Cloxacillin was ineffective against the three isolates and the two mixtures which were used in the antimicrobial susceptibility test which indicates once again that at least one of the bacteria with a role in the ulcerative process had not been isolated. Cloxacillin is a narrow spectrum penicillin type antibiotic which is primarily used against gram-positive cocci such as *Staphylococcus aureus* in humans or staphylococcal mastitis in cattle [Glasby 1979; Pratt & Fekety, 1986] and unlike penicillin G, is usually ineffective against gram-negative bacteria although it does have some degree of activity against gram-negative cocci [Russell, 1989]. A large gram-negative coccus was isolated from one ulcer but it has yet to be identified and possibly this may be one of the bacterial species responsible for the ulcerative process.

5.2.5.1.3 Amoxycillin

The animals which were pretreated with amoxycillin for three days prior to the acute dose of indomethacin ID₆₀ had very little ulceration in the small bowel but the little ulceration present was distributed throughout the small bowel, slightly more in the distal region (see fig. 5.1.1.3.1). This would suggest that amoxycillin was primarily effective against the aerobic bacteria although it did reduce ulceration in the ileum to some extent, when compared to the control animals. Amoxycillin was also effective in the antimicrobial susceptibility test against the unknown bacteria, Isolates 1, 2 and 3, although there appeared to be some resistant colonies. Amoxycillin works by inhibiting bacterial cell wall synthesis and is effective against *Streptococcus spp.*, *Enterococcus spp.*, *Salmonella spp.*, *Shigella spp.*, *Proteus mirabilis* and *Escherichia coli* [Neu, 1974; Pratt & Fekety, 1986].

5.2.5.1.4 Erythromycin

The animals which were pretreated for three days with erythromycin prior to the acute dose of indomethacin ID₆₀ had more ulceration within the small bowel than the control animals which had been dosed with distilled water. The majority of the ulceration was in the first half of the small bowel and the increase in ulceration was approximately of the same magnitude throughout when compared to the control animals (see fig. 5.1.1.3.1). It would appear that erythromycin killed one or more strains of bacteria which are responsible for keeping a healthy balance between the microflora within the small bowel [Simon & Gorbach, 1986, 1987]. In optimum conditions most coliform bacteria divide every 20 minutes but if this happened *in vivo* the host organism would soon be overwhelmed [Simon & Gorbach, 1986, 1987]. In the small bowel bacteria usually only replicate about four times a day [Gibbons & Kapsimalis, 1967] as other bacteria within the vicinity inhibit their growth [Simon & Gorbach, 1986, 1987]. In this instance it would appear that the bacteria responsible for the ulcerative process were able to multiply rapidly once the inhibiting strains were destroyed. Erythromycin is both bacteriostatic and bactericidal for susceptible organisms and acts by inhibiting protein synthesis by acting on the 50S unit of ribosomes [Mao & Putterman, 1969]. Erythromycin is primarily effective against gram-positive bacteria especially *Pneumococcus*, *Streptococcus spp.*, *Staphylococcus spp.* and *Enterococcus spp.*

Although it is effective against some gram-negative bacilli, such as *Bacteroides fragilis*, other gram-negative bacteria, notably the *Enterobacteriaceae*, are intrinsically resistant [Pratt & Fekety, 1986]. Erythromycin was ineffective against the three isolates and the two mixtures, which were used in the antimicrobial sensitivity test, that had been cultured in the Cooked Meat broth. This would appear to indicate that the bacteria which are susceptible to erythromycin are not associated with the small bowel ulcers and to confirm that they are not responsible for the ulcerative process.

5.2.5.1.5 Nalidixic Acid

The animals which were pretreated for three days with nalidixic acid prior to the acute dose of indomethacin ID₆₀ also had more ulceration within the small bowel than the control animals which had been dosed with distilled water. The ulceration was quite evenly distributed although it was greater in the latter half of the small bowel (see fig. 5.1.1.3.1). As with erythromycin it would appear that nalidixic acid killed one or more strains of bacteria which are responsible for maintaining a healthy balance between the microflora within the small bowel [Simon & Gorbach, 1986, 1987]. Nalidixic acid is unusual in that it is active against several different types of gram-negative bacteria whereas gram-positive organisms are mostly resistant [Deitz *et al.*, 1964; Russell, 1989]. Nalidixic acid rapidly and reversibly inhibits DNA replication, in susceptible bacteria, by binding to a subunit of the DNA gyrase enzyme [Sugino *et al.*, 1977; Gellert *et al.*, 1977]. Most strains of *Escherichia coli*, *Klebsiella*, *Enterobacter* and *Proteus* (*mirabilis*, *morganii*, *vulgaris*) are susceptible as are most species of *Salmonella* and *Shigella* [Brumfitt & Pursell, 1971; Stamey, 1971]. Bacteria that are resistant to nalidixic acid include most gram-positive cocci and anaerobic bacteria in general [Gleckman *et al.*, 1979]. Nalidixic acid was effective against all three isolates and both mixtures although there was evidence of resistant microorganisms as there were colonies growing within the zone of resistance for Isolates 1 and 2.

5.2.5.1.6 Neomycin Sulphate

The animals which were pretreated for three days with neomycin sulphate prior to the acute dose of indomethacin ID₆₀ also had less ulceration within the small bowel than the control animals which had been dosed with distilled water although this was not

significant. The ulceration was reduced in the first half of the small bowel, when compared to the control animals, whereas the degree of ulceration within the latter half of the small bowel was approximately the same (see fig. 5.1.1.3.1). This suggested that one or some of the bacteria involved in the ulcerative process were aerobes. Neomycin sulphate is bactericidal for many gram-positive and gram-negative bacteria and acts by binding to the 30S subunit of bacterial ribosomes [Davies, 1964] which irreversibly inhibits protein synthesis [Pestka, 1971]. Anaerobic bacteria are not susceptible because active uptake of the drug requires respiration (electron transport) [Brynan *et al.*, 1979] but bacteria which are susceptible include *Escherichia coli*, *Klebsiella spp.*, *Enterobacter spp.* and *Proteus spp.* [Pratt & Fekety, 1986]. Neomycin sulphate was effective against all three isolates and both mixtures (mostly under aerobic conditions) although there was evidence of resistant microorganisms as there were colonies growing within the zone of resistance for Isolates 1 and 2.

5.2.5.2 Combined Treatment

Animals pretreated with a combination of metronidazole and amoxycillin three days prior to an indomethacin acute (ID₆₀, ID₆₅ and ID₇₀) challenge did not exhibit any form of ulceration. The animals pretreated with the combined antibiotics a week or two weeks prior to the indomethacin acute ID₆₀ challenge were still protected from the ulcerative process to some degree although the effect of the antibiotics was beginning to wear off after two weeks. The distribution of ulceration within the small bowel of the animals which had received the antibiotics a week before the indomethacin challenge was equally distributed (see fig. 5.1.1.3.2). The animals which had been pretreated with combined antibiotics two weeks prior to the indomethacin challenge, however, had a distribution of ulceration which was similar to that seen in the control animals although still significantly ($p < 0.05$) less. This would appear to indicate that initially most of the small bowel flora, including those responsible for the ulcerative process, recovered from the antibiotic challenge at approximately the same rate. Two weeks after the combined antibiotics, however, the usual balance between the bacterial species was beginning to reassert itself with the majority of bacteria responsible for the ulcerative process being present in the first half of the small bowel. This probably occurs as a result of the presence of one species of microorganism radically affecting the proliferation of another.

Interactions between the indigenous microflora are the major regulatory factor in maintaining a healthy balance within the small bowel [Wolin, 1974; Savage, 1977; Mackowiak, 1982; Rolfe, 1984] and there are several mechanisms whereby bacteria may interact to promote or prevent the growth of other microorganisms [Simon & Gorbach, 1986, 1987]. Some coliform bacteria, which compete for carbon [Freter, 1962], can inhibit the growth of *Shigella flexneri* by depleting the surrounding area of the substrate necessary for its growth. Other bacteria produce metabolic end products, such as short chain fatty acids, which can inhibit bacterial proliferation [Mackowiak, 1982; Byrne & Dankert, 1979] in susceptible organisms such as *Shigella flexneri* [Hentges & Maier, 1970]. Alternatively other bacteria such as the facultative anaerobes can deplete an area of oxygen and thus create an environment in which obligate anaerobes can survive [Simon & Gorbach, 1986, 1987]. Some bacteria are even able to produce their own specific antibiotic-like substance such as bacitracin, which is produced by a strain of *Bacillus subtilis* [Meleney & Johnson, 1949], and colicins, which are produced by strains of *Escherichia coli* [Luckey, 1977], that inhibit the growth of other species or even control their own growth in an autoregulatory fashion [Simon & Gorbach, 1986, 1987].

5.2.6 Bacteria Which Could be Involved in the Formation of Ulcers

All these results would suggest that there is more than one species of bacteria responsible for the ulcerative process and that at least one of these bacteria is aerobic and another an anaerobe as it requires a combination of amoxycillin and metronidazole together to prevent the ulcerative process. *Escherichia coli* have already been implicated, as germ-free rats, mono-contaminated with this bacterium, are susceptible to indomethacin-induced ulceration [Robert & Asano, 1977], although this is less severe than that seen in conventional animals. Some of the bacteria responsible may also act together synergistically, as removing one or two of the bacterial participants in the ulcerative process, via treatment with certain individual antibiotics, leads to a significant decrease in ulceration. Three bacteria which could be responsible are *Bacteroides*, *Peptococcus*, and *Peptostreptococcus* which are already known to act synergistically [Morse, 1980]. These particular microorganisms are resistant to the antibiotic nalidixic acid, but susceptible to metronidazole, which could explain the increase in ulceration seen in the animals pretreated with nalidixic acid and the lack of ulceration with metronidazole.

Bacteroides spp., anaerobic gram-negative bacteria, are commensal organisms, but can cause severe, often life-threatening infections, mostly in proximity to the mucosal surfaces where they exist normally (as 'potential pathogens') [Sonnenwirth, 1980]. *Bacteroides fragilis* is frequently isolated from clinical cases of sepsis involving anaerobic gram-negative rods and accounts for the majority of infections within the peritoneal cavity [Gorbach & Bartlett, 1974; Polk & Kasper, 1977]. Abscess formation as a result of small or large bowel perforations (similar to those observed in rats dosed with indomethacin ID₁₂₅) are caused primarily by a major virulence factor of *Bacteroides fragilis*, the capsular polysaccharide complex (CPC). This surface associated protein is responsible for abscess formation in both the rat and mouse models of peritoneal sepsis [Onderdonk *et al.*, 1977; Shapiro *et al.*, 1982]. *Peptococcus spp.* and *Peptostreptococcus spp.*, anaerobic gram-positive bacteria, are also part of the indigenous flora of the gastrointestinal tract and are responsible for a variety of infections, mostly in synergy with other microorganisms. When in mixed infections with *Bacteroides spp.* the result is severe necrosis of the surrounding tissue [Morse, 1980], similar to that seen in the indomethacin-induced small bowel ulcer. Other bacteria which could have a role in ulcerogenesis are *Fusobacterium spp.*, gram-negative spindle-shaped rods, similar to those seen within the ulcers via SEM, which are obligate anaerobes. These bacteria are found within the gastrointestinal tract and have also been isolated from various purulent lesions, in both man and animals, as well as from tropical ulcers.

5.2.6.1 Possible Mechanisms by Which Bacteria May Facilitate Ulcerogenesis

One of the ways in which bacteria could facilitate the ulcerative process is via an inflammatory response caused by bacterial toxins permeating through the intestinal epithelium as a result of the increased permeability caused by indomethacin. Endotoxins, such as lipopolysaccharide, a large molecule in the outer wall of gram-negative bacteria is extremely proinflammatory and induces the inflammatory cytokine cascade [Dinarello, 1991]. Another bacterial product, N-formylmethionylleucylphenylalanine (FMLP) is a tripeptide known to be released by *Escherichia coli* during replication [Grisham & Granger, 1988]. FMLP is nontoxic to mammalian cells but is extremely chemotactic for neutrophils, activating them to release oxygen-derived free radicals [Tauber & Babior,

1985]. (The role of cytokines in indomethacin-induced ulceration will be discussed in the next chapter.)

Another way in which bacteria may mediate the ulcerative process is through their end products of metabolism. The intraluminal pH of germ-free animals is more alkaline than that of normal rats [Simon & Gorbach, 1986, 1987], colonised by the usual complement of gastrointestinal microflora, and the more acidic environment is probably due to the metabolic end products of the various bacteria. The family of bacteria called *Bacteroidaceae* are gram-negative bacilli which are obligate anaerobes and these bacteria outnumber coliforms in the gastrointestinal tract a 100 fold. Two genera, *Bacteroides* and *Fusobacterium*, both produce short chain fatty acids as by products of metabolism. Most *Bacteroides spp.* produce a combination of succinic, lactic, acetic, formic, propionic and isovaleric acid whereas some produce a mixture of butyric and acetic acid [Sonnenwirth, 1980; Holt *et al.*, 1994]. The *Fusobacterium spp.* yield butyric acid as a major product of metabolism from peptone or carbohydrates, often in conjunction with acetic and lactic acid, although occasionally with propionic, formic and succinic acid [Sonnenwirth, 1980; Holt *et al.*, 1994]. In addition to these bacteria the facultative anaerobes, such as *Enterobacter*, *Klebsiella*, *Proteus* and *Escherichia coli*, are known to produce acid and gas from a variety of carbohydrates [Sonnenwirth, 1980, Holt *et al.*, 1994]. Gram-positive anaerobic cocci such as *Peptostreptococcus* metabolise peptone, mainly to acetic acid [Holt *et al.*, 1994] and some aerobic bacteria also have volatile fatty acids as a by product of metabolism [Byrne & Dankert, 1979]. Thus the presence of these acidic bacterial by products must contribute towards the lowering of the pH within the gastrointestinal tract of conventional rats, when compared to germ-free animals, increasing the un-ionised proportion of NSAIDs.

Conceivably, in the areas immediately surrounding the small discrete ulcers, where the bacterial numbers have already begun to increase dramatically, the environmental pH may become significantly more acidic than that of the non ulcerated areas of small bowel. These ulcers are also full of bacteria, such as *Escherichia coli* and probably *Bacteroides spp.*, which are able to deconjugate the indomethacin conjugate [Lewis & Gorbach, 1972; Scheline, 1973; Plaa, 1975]; thus releasing the parent drug into a region where the

pH is probably low enough for a significant proportion of the drug to be un-ionised. This decrease in environmental pH may lead to even more of the NSAID being able to pass through the lipid bilayer into the cells of the surrounding epithelium, where it becomes trapped, exacerbating the initial damage and resulting in an increase in size of the original ulcer. (Indomethacin is known to be one of the most toxic NSAIDs to cultures of intestinal epithelial cells [Allen *et al.*, 1991; Yamada *et al.*, 1992] and this toxicity is enhanced as the pH is reduced from 7.4 to 6.0 [Chmisse *et al.*, 1994].) This process may continue, with a large proportion of the deconjugated drug passing through the small bowel wall into the systemic circulation as absorption from the small bowel is also increased with the lowering of the intraluminal pH (see table 5.2.6.1). After reabsorption the NSAID is returned to the liver, remetabolised and excreted once again in the bile. Thus, as a consequence of the increase in bacterial numbers in conjunction with a lowering of the environmental pH, the enterohepatic recirculation of a single dose of indomethacin may be prolonged and far exceed the length of time generally expected. As the size of the ulcers and the numbers of bacteria within them increase, the area of small bowel with a lower pH becomes larger and eventually the individual ulcers coalesce. This could explain the longitudinal ulceration seen with high acute doses of indomethacin and the extended ulcers seen in the animals chronically dosed with diclofenac. This could also explain why, in severely ulcerated animals, a few discrete ulcers occasionally appear in the terminal ileum. The bacterial overgrowth, within the rest of the small bowel, may cause the pH to drop within the terminal ileum resulting in a significant proportion of the unconjugated drug being un-ionised in this region and being absorbed as a consequence.

Small Bowel Contents pH	NSAID pKa				
	Diclofenac 3.9	Naproxen 4.2	Indomethacin 4.5	Fenbufen 5.7	Piroxicam 6.3
7.00	0.40	0.40	0.40	0.41	0.44
6.75	0.22	0.22	0.22	0.24	0.28
6.50	0.13	0.13	0.13	0.14	0.19
6.25	0.07	0.07	0.07	0.09	0.14
6.00	0.04	0.04	0.04	0.06	0.11

Table 5.2.6.1 Predicted Gut/Plasma Ratios Calculated by the Henderson-Hasselbalch Equation

5.2.6.2 Effect of Variations in Composition of Intestinal Microflora

The degree and distribution of ulceration within the small bowel appears to be dependent on the bacterial species which may be colonising it at the time and on their numbers. There is a balance between protective and pathogenic bacteria with regard to the ulcerative process and if this balance is disturbed the severity of ulceration increases as a result. This finding probably explains the difference in response to the NSAIDs seen between the Wistar rats purchased from Interfauna and the Wistar rats bred by the Animal House at the University of Bath. At the beginning of the project, the rats from Interfauna were more resistant to ulcer formation because the balance of microflora within their gastrointestinal tract was different to that present in the Bath strain and probably had a higher ratio of protective species of bacteria to ulcerogenic ones. The original Bath strain were more susceptible to ulceration as the ratio of ulcerogenic bacteria present was probably higher and in fact at that time the Bath strain were susceptible to diarrhoea. This indicates that these rats already had greater numbers of bacteria within the gastrointestinal tract than would be expected and so the increase in bacterial numbers, in response to the dose of indomethacin, occurred more rapidly. The difference between the Interfauna rats and the Bath strain, seen at a later date, was probably due to the Bath animals having been bred from new healthier breeding stock and the Interfauna animals probably being colonised by a microflora of different population proportions to that found previously.

The balance between the populations of colonising bacteria would appear to differ between rats of the same strain over a period of time and the new strain of Bath University rats did appear to become more susceptible to ulceration after a few months duration. The differences in the balance between the gastrointestinal microflora could be due to a number of reasons, cross contamination with bacteria from other rat strains within the animal house being one of them. The most probable reason, however, is that this change was due to the food that the rats were fed as the Bath University strain (new breeding stock) had originally been supplied by Interfauna. It has been reported that a change of diet can affect the balance between the indigenous microflora within the gastrointestinal tract [Sharma *et al.*, 1995] and also alter their metabolic activity [Simon & Gorbach, 1986, 1987].

Interestingly, it has also been reported that starving a rat for 24 hours can lead to a variable and sometimes extensive alteration in the numbers of some of the indigenous gastrointestinal microflora within the small bowel [Smith, 1965; Stephen, 1985; Butzler, 1987]. This could possibly explain the anomalous result when the second batch of Interfauna rats, which had been given an acute indomethacin ID₇₅ dose after a 21 hour fast, had very little ulceration. Under these circumstances the ulceration would have been expected to be more severe than that observed in the animals allowed free access to food before and after dosing. After the 21 hour fast there could have been a significant decrease of the bacterial species responsible for ulceration within the small bowel of the Interfauna rats (second batch) so that there were insufficient numbers available for ulcerogenesis after ingestion of the indomethacin ID₇₅ dose. Thus within the animals given the lower dose of indomethacin the physiological repair processes were probably able to rectify the topical damage before sufficient bacteria were available to initiate an inflammatory response. After administration of the indomethacin ID₁₂₅ dose to Interfauna rats (second batch) following a 21 hour fast, however, the repair processes were probably delayed by the increased inhibition of cyclooxygenase (COX-1 and COX-2). This in conjunction with the increase in enterohepatic recirculation of the drug may have given the bacteria enough time in which to multiply to sufficient numbers thus enabling them to initiate an inflammatory response.

6.0 RESULTS AND DISCUSSION - ROLE OF CYTOKINES

6.1 RESULTS - ROLE OF CYTOKINES IN ULCER FORMATION

6.1.1 Anti-Mouse TNF- α

6.1.1.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.1.1.1 Donated Polyclonal Rabbit Anti- Mouse TNF- α in Hyperimmune Serum

6.1.1.1.1.1 Positive Control Tissue - Activated Rat Lung

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the donated polyclonal anti-mouse TNF- α (1/50, 1/100, 1/200, 1/500 and 1/1000) with and without the trypsin antigen retrieval step. Optimum binding was found to occur after the trypsin step so for this antibody it was decided to include this antigen retrieval step every time. The 1/100 dilution of the antibody gave the optimum degree of staining, however, when the experiment was repeated using control rabbit serum, which had been obtained from a control rabbit (Animal House (AH), Bath University), a high degree of staining was found at the equivalent dilution. As a consequence it was decided that the antibody should be used at a dilution of 1/200 as at this dilution the staining obtained with the control rabbit (AH) serum was visibly less than that found in the sections treated with the equivalent amount of antiserum.

Unfortunately, there was still a noticeable degree of binding to the tissue by the control rabbit (AH) serum. (Increasing the dilution of the antiserum and the control serum had lead to there being no visible staining with either of the sera.) No staining had been found in the negative control sections, treated as the other sections except that they had been incubated with buffer only during the primary incubation step, (when the other sections had been incubated with either antiserum or control serum) which illustrated that there was not any non-specific binding. Therefore the binding of the control rabbit (AH) serum to the tissue was specific. (The rabbit from the Animal House had probably developed antibodies against the rats which also shared the complex.)

6.1.1.1.2 Experimental Ulcerated Rat Small Bowel Tissue - Indomethacin ID₆₀

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the donated polyclonal anti-mouse TNF- α (1/50, 1/100, 1/200, 1/500 and 1/1000) as described above, for the control activated lung tissue, with the same result. The binding of the control rabbit serum to the small bowel tissue was less than that seen in the sections treated with the antiserum, but the staining was still quite strong in the region of the ulcer, even at a 1/200 dilution. At this time it was decided that a commercially available anti-mouse TNF- α might provide better results.

6.1.1.1.2 Genzyme Polyclonal Rabbit Anti- Mouse TNF- α in Hyperimmune Serum

6.1.1.1.2.1 Positive Control Tissue - Activated Rat Lung

Genzyme was the only company that had antibodies available, reported as cross reacting with the relevant rat protein, which were also suitable for immunohistochemistry [Diamond *et al.*, 1991; Merrill *et al.*, 1993; Teti *et al.*, 1993]. Sections of activated lung were titrated as already described. As before, the staining was greater in the sections which had been trypsinised first. The rabbit control serum used for comparison with this antisera had been purchased from Sigma. However, at the same dilution the control rabbit serum {Sigma} displayed approximately twice the staining found in the sections treated with the polyclonal anti-mouse TNF- α {Genzyme}. The company was contacted and after a visit from their representatives an alternative sample of the antibody was supplied. However, a similar result was found when using the substitute antiserum.

6.1.1.1.2.2 Experimental Ulcerated Rat Small Bowel Tissue - Indomethacin ID₆₀

These sections were processed as described above in section 6.1.1.1.2.1.

6.1.1.1.3 Donated Polyclonal Rabbit Anti- Mouse TNF- α (IgG Fraction)

6.1.1.1.3.1 Positive Control Tissue - Activated Rat Lung

The original antiserum, which was a gift from Dr Steven Kunkel, was quite old (approximately 5 years since exsanguination) so it was suggested [Watson, 1995] that an improvement in specificity might be obtained using the IgG fraction of the antiserum. As a result a partial antiserum fractionation was carried out on the antiserum and the control

serum. The volume of the resultant IgG fractions was approximately 2.5 times that of the original volumes of the antiserum and the control rabbit (AH) serum. The tissue sections were titrated against the IgG fractions of the donated polyclonal anti-mouse TNF- α and the control rabbit (AH) serum but at dilutions which had been corrected to account for the increase in volume (1/20, 1/40, 1/80, 1/200 and 1/400). As expected the 1/80 dilution was found to be the most suitable. At this concentration the IgG fraction of the anti-mouse TNF- α bound well, producing good staining (see photo. 6.1.4a {A & B}), whereas the tissue which had been incubated with the IgG fraction of the control rabbit (AH) serum displayed very little staining at all (see photo. 6.1.4a {C}). All further immunohistochemical experiments, investigating the presence of TNF- α in tissue sections, were conducted using this antiserum.

6.1.1.1.3.2 Experimental Ulcerated Rat Small Bowel Tissue - Indomethacin ID₆₀

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the IgG fraction of the donated polyclonal anti-mouse TNF- α (1/20, 1/40, 1/80, 1/200 and 1/400) as described above. As expected the 1/80 dilution was found to be the most suitable. At this concentration the IgG fraction of the anti-mouse TNF- α bound well producing good staining (see photo. 6.1.1a) whereas the tissue which had been incubated with the RyG fraction of the control rabbit (AH) serum displayed very little staining at all (see photo. 6.1.1b {C & D}). This result confirmed that all further immunohistochemical experiments, investigating the presence of TNF- α in tissue sections, should be conducted using this antiserum at this dilution. The degree of antibody staining in this tissue was very slight in the unulcerated regions of the small bowel tissue, between the discrete ulcers (see photo. 6.1.1a {C & D}), whereas the staining was very strong in the region of the Peyer's patch associated with the ulcer (see table 6.1.1.1.3 and photo. 6.1.1a {A & B}). The staining was quite evenly distributed throughout the tissue except within the Peyer's patches where individual cells appeared to be positively stained for TNF- α .

6.1.1.1.3.3 Experimental Unulcerated Rat Small Bowel Tissue - Indomethacin ID₃₀

Sections were incubated with the IgG fraction of the donated polyclonal antibody, after the trypsin antigen retrieval step, at a dilution of 1/80 (1/200). The degree of staining

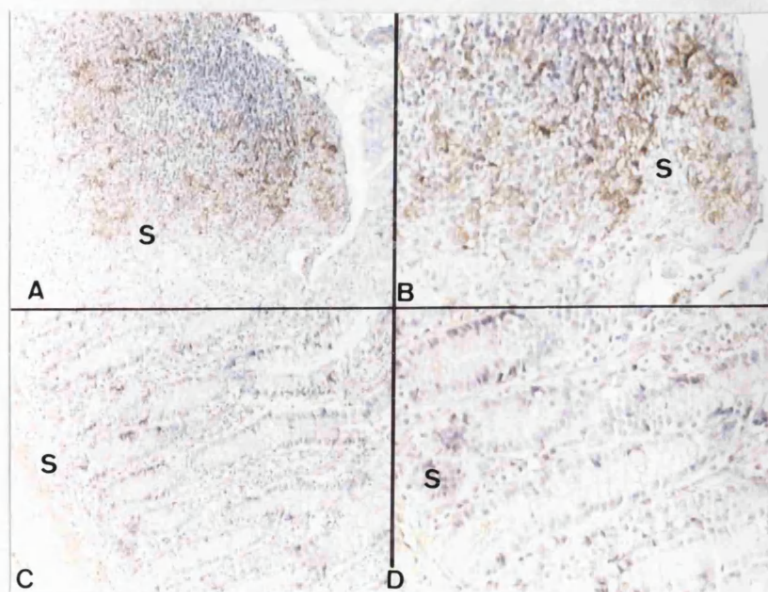


Photo. 6.1.1a

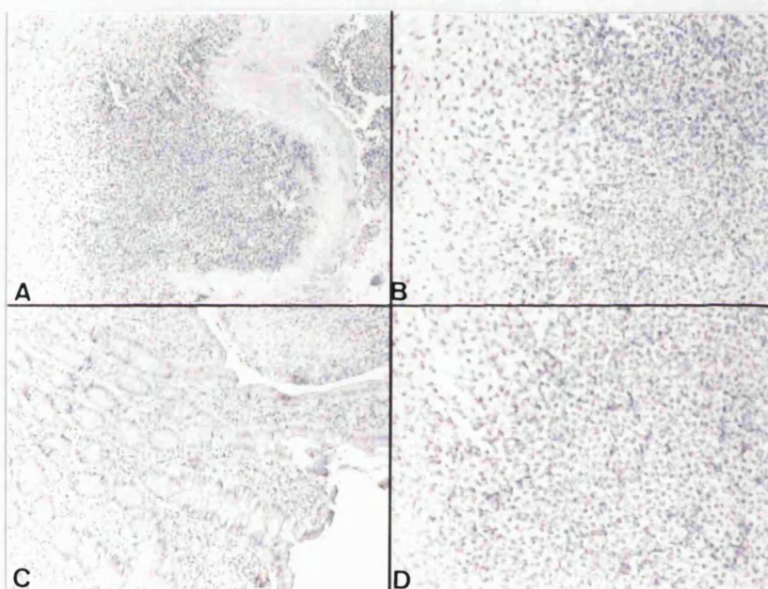


Photo. 6.1.1b

Photo. 6.1.1 FFPE Small Bowel Tissue From Rats Dosed Once, Orally, With Indomethacin ID₆₀ (6.0 mg/kg)

a. Ulcerated Rat Small Bowel Tissue Incubated With Polyclonal Anti-Mouse TNF- α (IgG Fraction). Brown Staining (S) Indicates the Presence of TNF- α

A & B = Intensely stained Peyer's patch indicates increased levels of TNF- α {A \times 60 & B \times 120}

C & D = Lightly stained un ulcerated region (between ulcers) indicates decreased levels of TNF- α .{C \times 60 & D \times 120}

b. Ulcerated Rat Small Bowel Tissue Incubated Without Anti-Mouse TNF- α (Primary Antibody)

A & B = Peyer's patch incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {A \times 60 & B \times 120}

C & D = Peyer's patch incubated with control rabbit (AH) serum (IgG fraction) instead of primary antibody (Serum Control). Lack of significant positive staining indicates that binding of the rabbit serum (IgG fraction) to other antigens within this tissue is insignificant at the dilution used for the primary antibody {C \times 60 & D \times 120}

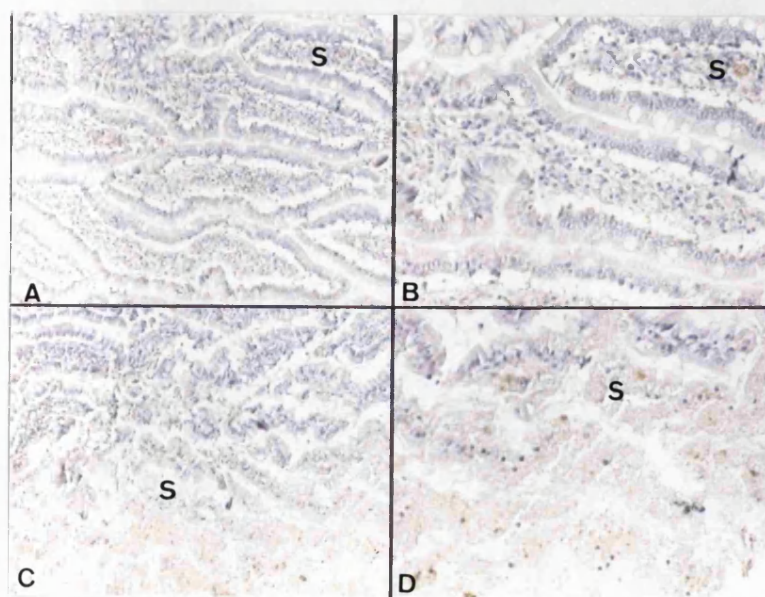


Photo. 6.1.2

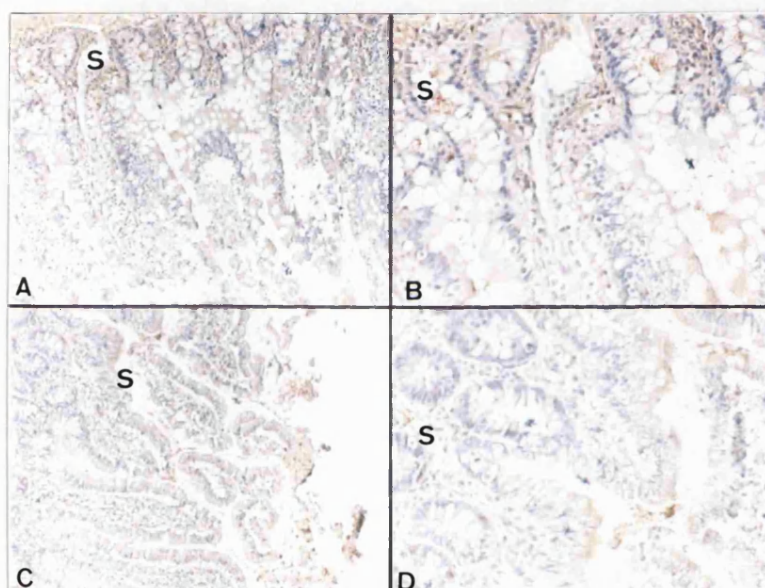


Photo. 6.1.3

Photo. 6.1.2 FFPE Small Bowel Tissue From Rats Dosed Once, Orally, With Indomethacin ID₃₀ (3.0 mg/kg) (Non Ulcer Causing Dose)

Unulcerated Rat Small Bowel Tissue Incubated With Polyclonal Anti-Mouse TNF- α (IgG Fraction). Brown Staining (S) Indicates the Presence of TNF- α

A & B = Lightly stained small bowel tissue indicates reduced levels of TNF- α when compared with normal control tissue, but more than that observed in un ulcerated regions (between ulcers) in tissue taken from rats with ulcers {A \times 60 & B \times 120}

C & D = As above {C \times 60 & D \times 120}

Photo. 6.1.3 FFPE Rat Small Bowel Control Tissues Incubated With Polyclonal Anti-Mouse TNF- α (IgG Fraction). Brown Staining (S) Indicates the Presence of TNF- α

A & B = Moderately stained un ulcerated control tissue indicates that there are significant levels of TNF- α within the normal rat small bowel {A \times 60 & B \times 120}

C & D = Light/Moderately stained vehicle (10% Tween-80) control rat small bowel tissue indicates reduced levels of TNF- α when compared with normal control tissue, but more than that observed in tissue from animals dosed with indomethacin ID₃₀ (3.0 mg/kg) {C \times 60 & D \times 120}

was slightly greater than that seen in the unulcerated region of the ulcerated ID₆₀ small bowel tissue (see table 6.1.1.1.3 and photo. 6.1.2).

6.1.1.1.3.4 Experimental Unulcerated Rat Small Bowel Tissue - Vehicle Control

Sections were incubated with the IgG fraction of the donated polyclonal antibody, after the trypsin antigen retrieval step, at a dilution of 1/80 (1/200). The degree of staining was greater than that seen in the unulcerated region of the ulcerated ID₆₀ small bowel tissue and slightly more than that seen in the ID₃₀ small bowel tissue (see table 6.1.1.1.3 and photo. 6.1.3 {C & D}).

6.1.1.1.3.5 Experimental Unulcerated Rat Small Bowel Tissue - Control

Sections were incubated with the IgG fraction of the donated polyclonal antibody, after the trypsin antigen retrieval step, at a dilution of 1/80 (1/200). The degree of staining was greater than that seen in the unulcerated region of the ulcerated ID₆₀ small bowel tissue and the ID₃₀ small bowel tissue (see table 6.1.1.1.3 and photo. 6.1.3 {A & B}).

6.1.2 Genzyme Polyclonal Rabbit Anti- Mouse IL-1 α in Hyperimmune Serum

6.1.2.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.2.1.1 Positive Control Tissue - Activated Rat Lung

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the polyclonal anti-mouse IL-1 α (1/50, 1/100, 1/200, 1/500 and 1/1000) with and without the trypsin antigen retrieval step. Optimum binding was found to occur in the sections which were not treated with trypsin so for this antibody it was decided not to include this antigen retrieval step. The 1/500 dilution of the antibody gave the optimum degree of staining (see photo. 6.1.4b {A & B}) and the sections treated with the control rabbit serum {Sigma} at the same dilution had no staining at all (see photo. 6.1.4b {C}).

6.1.2.1.2 Experimental Ulcerated Rat Small Bowel Tissue - Indomethacin ID₆₀

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the polyclonal anti-mouse IL-1 α (1/50, 1/100, 1/200, 1/500 and 1/1000) with and without

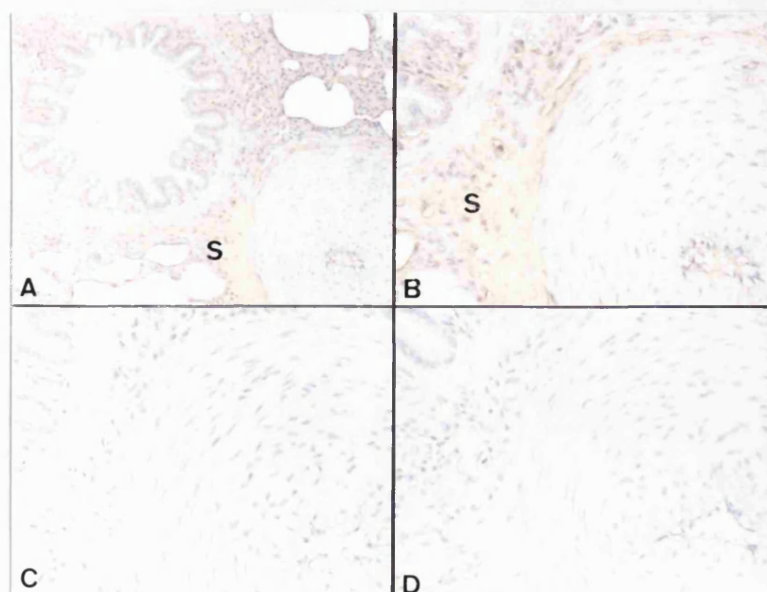


Photo. 6.1.4a

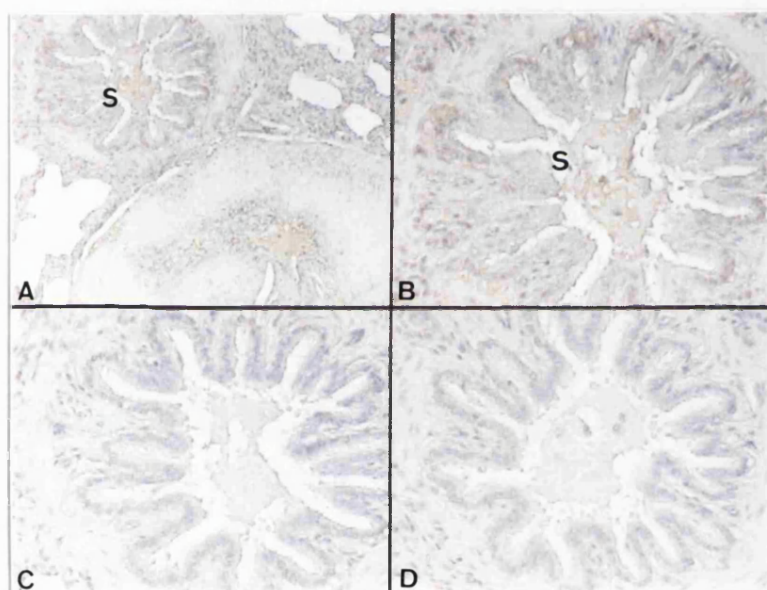


Photo. 6.1.4b

Photo. 6.1.4 FFPE Activated (Incubated with LPS for 1 hr at 37°C) Rat Lung Used as Positive Control Tissue for Both the Polyclonal Anti-Mouse TNF- α (IgG Fraction) and the Polyclonal Anti-Mouse IL-1 α .

- a. Positive Control Tissues For Polyclonal Anti-Mouse TNF- α (IgG Fraction). Brown Staining (S) Indicates the Presence of TNF- α

A & B = Activated rat lung incubated with polyclonal anti-mouse TNF- α (IgG fraction). Moderate staining indicates that the polyclonal anti-mouse TNF- α has bound to the TNF- α within the activated rat lung tissue demonstrating that the antibody has bound successfully {A \times 60 & B \times 120}

C = Activated rat lung incubated with control rabbit (AH) serum (IgG fraction) instead of primary antibody (Serum Control). Lack of significant positive staining indicates that binding of the rabbit serum (IgG fraction) to other antigens within this tissue is insignificant at the dilution used for the primary antibody {C \times 120}

D = Activated rat lung incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {D \times 120}

- b. A Above But For The Polyclonal Anti-Mouse IL-1 α .

Rat Small Bowel Tissue				
Antibody	Indomethacin ID ₆₀ (Ulcers)	Indomethacin ID ₃₀ (No Ulcers)	Vehicle 10 % Tween-80	Control
Anti-Mouse TNF- α	X (Peyer's Patches) (XXXXX)	XX	XX	XXX

Table 6.1.1.1.3 Comparison of Immunohistochemical Staining With Anti-Mouse TNF- α in Experimental Rat Small Bowel Tissue

Rat Small Bowel Tissue				
Antibody	Indomethacin ID ₆₀ (Ulcers)	Indomethacin ID ₃₀ (No Ulcers)	Vehicle 10 % Tween-80	Control
Anti-Mouse IL-1 α	X	XXXXXX	XXX	XXX

Table 6.1.2 Comparison of Immunohistochemical Staining With Anti-Mouse IL-1 α in Experimental Rat Small Bowel Tissue

XXXXXX = Most Staining
X = Least Staining

the trypsin antigen retrieval step, as with the positive control tissue. As before, optimum binding was found in the sections which were not treated with trypsin, this confirmed that this antibody should be used without the trypsin antigen retrieval step. The 1/500 dilution of the antibody gave the optimum degree of staining and the sections treated with the control rabbit serum {Sigma} at the same dilution had no staining at all (see photo. 6.1.5b {C & D}). However, there was some degree of background staining in the small bowel tissue sections treated with the antiserum. As a result it was decided to experiment with the conditions under which the immunohistochemical procedure was carried out. The incubation of the tissue with antibody was compared at 30, 45 and 60 minutes, at 37°C and at room temperature, and the incubations with the secondary antibody and the ABComplex were similarly shortened. After experimenting with the incubation times and temperature it was found that optimum staining occurred when following the basic protocol but with the incubation time of the secondary antibody reduced from 40 minutes to 30 minutes (see photo. 6.1.5a). Thus all tissue sections treated with this antibody were processed accordingly (including the control sections).

The degree of staining was less in the ulcerated small bowel tissue from animals which had been dosed with indomethacin ID₆₀ than in the other experimental small bowel tissue (see table 6.1.2 and photo. 6.1.5a {A & B}). However, in areas of this tissue which were totally free of ulceration (see photo. 6.1.5a {C & D}) the staining was slightly more intense than that seen in tissue adjacent to (see photo. 6.1.5a {A & B}) and between the ulcers (see photo. 6.1.6 {A & B}). The ulcers were associated with Peyer's patches and the area of necrosis, which was surrounded by neutrophils, included the adjacent tissue as well as the lymphoid follicle and extended into the muscle layer. The lamina propria was full of inflammatory cells (lymphocytes, macrophages and plasma cells) as was the mesentery (macrophages, lymphocytes and neutrophils) and the muscle layer was itself the centre of an inflammatory response. In other areas of this tissue there was evidence of erosions above active Peyer's patches and away from the ulcerated areas normal Peyer's patches were visible. In areas of small bowel tissue away from Peyer's patches there was evidence of microscopic erosions which were not associated with any inflammatory cells.

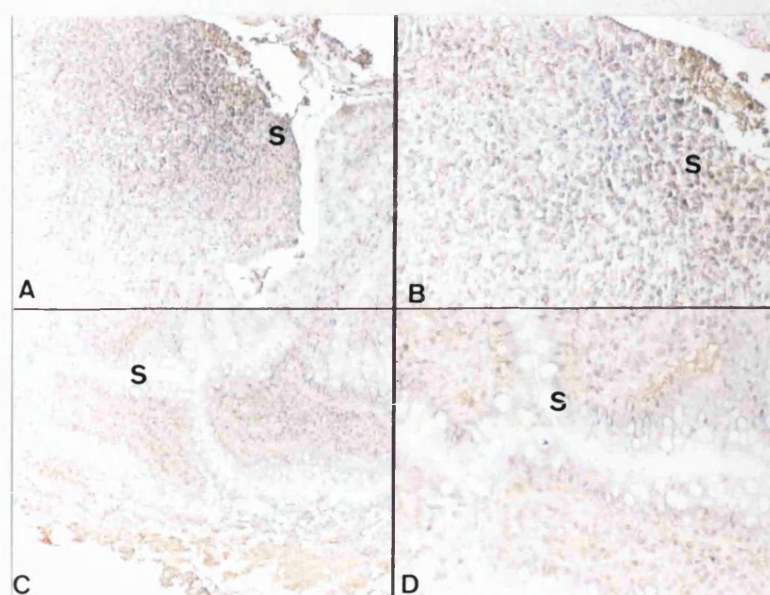


Photo. 6.1.5a

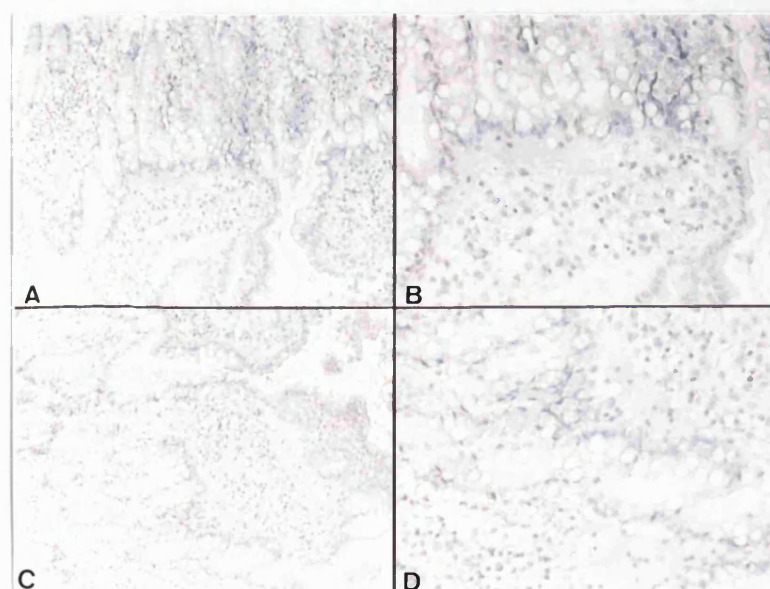


Photo. 6.1.5b

Photo. 6.1.5 FFPE Small Bowel Tissue From Rats Dosed Once, Orally, With Indomethacin ID₆₀ (6.0 mg/kg)

- a. Ulcerated Rat Small Bowel Tissue Incubated With Polyclonal Anti-Mouse IL-1 α . Brown Staining (S) Indicates the Presence of IL-1 α

A & B = Lightly stained Peyer's patch indicates decreased levels of IL-1 α {A \times 60 & B \times 120}

C & D = Light/moderately stained un ulcerated region (away from ulcers) indicates decreased levels of IL-1 α when compared with control and vehicle control tissues {C \times 60 & D \times 120}

- b. Ulcerated Rat Small Bowel Tissue Incubated Without Anti-Mouse IL-1 α (Primary Antibody)

A & B = Peyer's patch incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {A \times 60 & B \times 120}

C & D = Peyer's patch incubated with control rabbit (AH) serum instead of primary antibody (Serum Control). Lack of significant positive staining indicates that binding of the rabbit serum to other antigens within this tissue is insignificant at the dilution used for the primary antibody {C \times 60 & D \times 120}

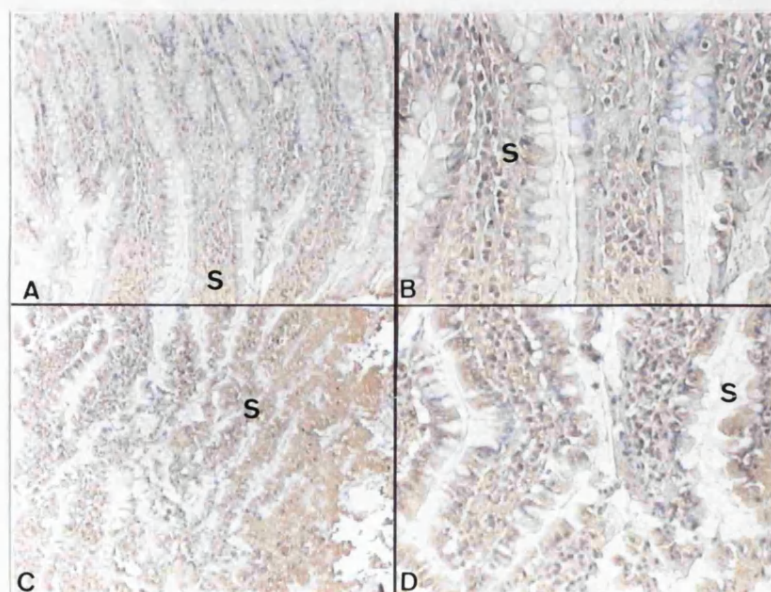


Photo. 6.1.6

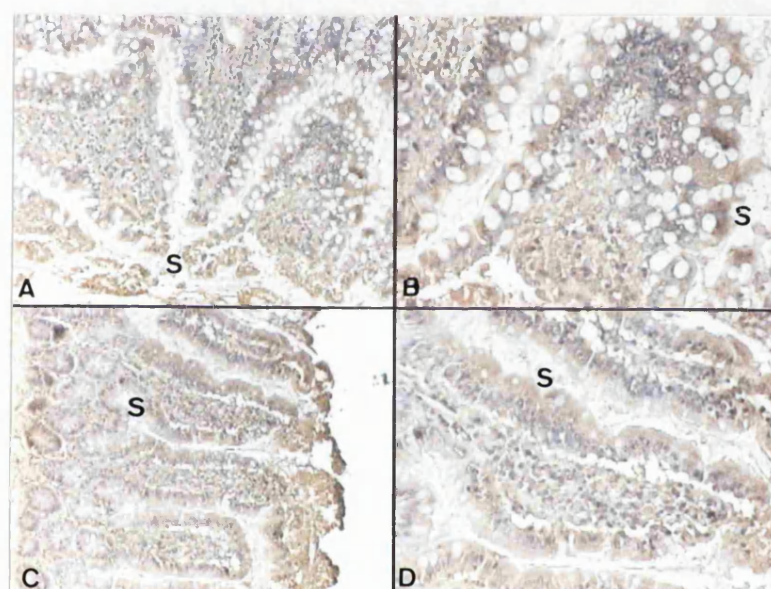


Photo. 6.1.7

Photo 6.1.6 FFPE Un ulcerated Rat Small Bowel Tissue Incubated With Polyclonal Anti-Mouse IL-1 α .
Brown Staining (S) Indicates The Presence of IL-1 α .

A & B = Small bowel tissue from rats dosed once, orally, with indomethacin ID₆₀ (6.0 mg/kg). Lightly stained un ulcerated region (between ulcers) indicates decreased levels of IL-1 α when compared with control and vehicle control tissues {A \times 60 & B \times 120}
C & D = Small bowel tissue from rats dosed once, orally, with indomethacin ID₃₀ (3.0 mg/kg). (Non ulcer causing dose). Densely stained small bowel tissue indicates increased levels of IL-1 α

Photo. 6.1.7 FFPE Rat Small Bowel Control Tissues Incubated With Polyclonal Anti-Mouse IL-1 α .
Brown Staining (S) Indicates the Presence of IL-1 α

A & B = Moderately stained un ulcerated control tissue indicates that there are significant levels of IL-1 α within the normal rat small bowel {A \times 60 & B \times 120}
C & D = Moderately stained vehicle (10% Tween-80) control rat small bowel tissue indicates that there are significant levels of IL-1 α , equivalent to that found within the control rat tissue {C \times 60 & D \times 120}

6.1.2.1.3 Experimental Unulcerated Rat Small Bowel Tissue - Indomethacin ID₃₀

Tissue sections were incubated as above with the polyclonal anti-mouse IL-1 α , without the antigen retrieval step, at a dilution of 1/500. There was a high degree of staining and these sections were the most densely staining small bowel tissue (see table 6.1.2 and photo. 6.1.6 {C & D}). Although there was no sign of ulceration within this tissue and the Peyer's patches were normal in appearance, the lamina propria contained many mononuclear cells (lymphocytes, macrophages and plasma cells) far more than in the lamina propria of the control tissues. There was no evidence of any inflammatory response within the muscle layer. Individual cells of the epithelium covering the villi were positively stained for IL-1 α , almost twice as many as observed in the control tissues, with the epithelium on the tips staining more than the epithelium in the crypts.

6.1.2.1.4 Experimental Unulcerated Rat Small Bowel Tissue - Vehicle Control

Tissue sections were incubated as above with the polyclonal anti-mouse IL-1 α , without the antigen retrieval step, at a dilution of 1/500. There was a good degree of staining which was approximately half that seen in the un ulcerated small bowel tissue from animals dosed with indomethacin ID₃₀, but double that seen in the slightly stained ulcerated bowel tissue (see table 6.1.2 and photo. 6.1.7 {C & D}). The mesentery of the control tissue was empty of cells, the lamina propria contained few mononuclear cells and the Peyer's patches were normal in appearance. Individual cells of the epithelium covering the villi stained positive for IL-1 α although staining was absent from goblet cells.

6.1.2.1.5 Experimental Unulcerated Rat Small Bowel Tissue - Control

Tissue sections were incubated as above with the polyclonal anti-mouse IL-1 α , without the antigen retrieval step, at a dilution of 1/500. There was a good degree of staining which was approximately equal to that seen in the small bowel tissue from the animals dosed with vehicle (Tween-80 1:9 distilled water) (see table 6.1.2 and photo. 6.1.7 {A & B}). The mesentery of the control tissue was empty of cells, the lamina propria contained few mononuclear cells and the Peyer's patches were normal in appearance. Individual

cells of the epithelium covering the villi stained positive for IL-1 α although staining was absent from goblet cells.

6.1.3 Genzyme Monoclonal Hamster Anti- Mouse IL-1 β

6.1.3.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.3.1.1 Positive Control Tissue - Activated Rat Spleen

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the monoclonal anti-mouse IL-1 β (1/50, 1/100, 1/200, 1/500 and 1/1000) with and without the trypsin antigen retrieval step. There was no visible staining so the process was repeated using the antibody at a higher concentration (1/10, 1/20, 1/30, 1/50 and 1/100) but without success. Initially it was thought that the monoclonal antibody was binding to the inside of the polystyrene LP3 tubes so these were replaced with polypropylene LP3 tubes and 1.0 per cent (w/v) bovine serum albumin was added to the buffer as a carrier protein. There was still no evidence of staining so it was decided to microwave the sections in order to try and improve the amount of antigen in the tissue. After this step there was some degree of staining but this was also visible in the negative control sections and it turned out to be the result of the DAB binding to endogenous peroxidase which had become 'unquenched' during the microwaving procedure. The immunohistochemical procedure was repeated again using the microwave antigen retrieval step but with the peroxidase quenching step after the microwaving procedure rather than prior to it. On this occasion there was no evidence of staining. It was then decided to use the pressure cooker technique to try and unmask any of the antigen which might have been present but this time the monoclonal anti-mouse IL-1 β bound to all the sections, as with the microwaving technique, because the endogenous peroxidase activity in the tissue had become 'unquenched'. The immunohistochemical procedure was repeated, again using the pressure cooker antigen retrieval step but with the peroxidase quenching step after the pressure cooking procedure rather than prior to it but on this occasion there was no evidence of staining. At this point it was decided to use frozen sections.

6.1.3.1.2 Experimental Ulcerated Rat Small Bowel Tissue - Indomethacin ID₆₀

These sections were processed as described above in section 6.1.3.1.1.

6.1.3.2 Frozen Sections

6.1.3.2.1 Positive Control Tissue - Activated Rat Spleen

Frozen sections taken from activated spleen tissue were titrated against the monoclonal hamster anti-mouse IL-1 β (1/50, 1/100, 1/200, 1/500 and 1/1000), however, there was a lot of staining in the negative control sections. After experimenting to determine the nature of the non-specific binding it was found to be a combination of the secondary antibody binding to the spleen tissue and also the DAB binding to endogenous peroxidase which had been insufficiently quenched. The problem of the non-specific binding of the secondary antibody was solved by using the biotinylated F(ab')₂ fragment of the rabbit anti-hamster immunoglobulins rather than the total IgG fraction. The F(ab')₂ fragments are more suitable with frozen sections as it avoids the binding that can occur between the Fc portion of the reagent and cell-surface Fc receptors, and spleen tissue is particularly susceptible to this type of non-specific binding. The problem of the DAB binding to the endogenous peroxidases was solved by increasing the concentration of hydrogen peroxide used in the quenching step from 0.6 per cent (v/v) to 2.5 per cent (v/v).

Frozen sections taken from activated spleen tissue were again titrated against the monoclonal hamster anti-mouse IL-1 β (1/50, 1/100, 1/200, 1/500 and 1/1000), under the new conditions, however, this time there was no staining visible in any of the tissue sections. The frozen sections were again titrated against the antibody, this time at different dilutions (1/10, 1/20, 1/30, 1/50 and 1/100) but still there was no evidence of the antibody binding to the tissue. The antibody had been reported to bind to frozen sections of rat kidney glomeruli, at a concentration of 50 μ g/ml (1/20 dilution) [Wu *et al.*, 1994], so it had been expected that the antibody would have bound to the rat spleen tissue under these experimental conditions. An attempt was made to increase the amount of antigen in the rat tissue by permeabilising the tissue with 0.1 per cent (v/v) saponin in tris buffered saline, pH 7.6 prior to the immunohistochemical procedure but still there was an absence of binding. At this stage it was decided to stop using this particular

antibody. Unfortunately all laboratory work ceased before an alternative antibody could be found.

6.1.4 Genzyme Monoclonal Mouse Anti-Human IL 4

6.1.4.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.4.1.1 Positive Control Tissue

6.1.4.1.1.1 - Reactive Human Tonsil

These sections were processed as described in section 6.1.3.1.1 (for the monoclonal hamster anti-mouse IL-1 β) with a similar lack of success. At this point it was decided to try the anti-human IL-4 on bowel tissue from a patient with ulcerative colitis as this tissue is reported as containing high levels of IL-4 [Sartor, 1994].

6.1.4.1.1.2 Bowel Tissue From Patient With Ulcerative Colitis

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the monoclonal anti-human IL-4 (1/50, 1/100, 1/200, 1/500 and 1/1000) with and without the trypsin antigen retrieval step. There was no visible staining so at this point it was decided to use frozen sections.

6.1.4.1.2 Experimental Ulcerated Rat Small Bowel Tissue - Indomethacin ID₆₀

These sections were processed as described above in section 6.1.4.1.1.1.

6.1.4.2 Frozen Sections

6.1.4.2.1 Positive Control Tissue - Reactive Human Tonsil

Frozen sections taken from reactive human tonsil tissue were titrated against the monoclonal mouse anti-human IL-4 (1/50, 1/100, 1/200, 1/500 and 1/1000), however, there was no staining visible. The frozen sections were again titrated against the antibody, this time at different dilutions (1/12.5, 1/25, 1/50 and 1/100) but still there was no evidence of the antibody binding to the tissue. The antibody had been reported to bind to frozen sections of rat brain [Khoury *et al.*, 1992] and rat heart [Hancock *et al.*, 1993], so it had been expected that the antibody would have bound to the human tissue

under the experimental conditions tried. An attempt was made to increase the amount of antigen in the tonsil tissue by permeabilising the tissue with 0.1 per cent (v/v) saponin in tris buffered saline, pH 7.6 prior to the immunohistochemical procedure but still there was an absence of binding. At this stage it was decided to stop using this particular antibody until a suitable control tissue or an alternative antibody could be found. Unfortunately all laboratory work ceased before either alternative could be found.

6.1.5 Genzyme Polyclonal Rabbit Anti-Human TNF- α in Hyperimmune Serum

6.1.5.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.5.1.1 Positive Control Tissue - Reactive Human Tonsil

Sections taken from formalin-fixed paraffin-embedded tissue were titrated against the polyclonal anti-human TNF- α (1/50, 1/100, 1/200, 1/500 and 1/1000) with and without the trypsin antigen retrieval step. There was no visible staining so the process was repeated using the antibody at a higher concentration (1/12.5, 1/25, 1/50 and 1/100). There was still no evidence of staining so it was decided to microwave the sections in order to try and improve the amount of antigen in the tissue. After this step there was some degree of staining but this was also visible in the negative control sections and it turned out to be the result of the DAB binding to endogenous peroxidase which had become 'unquenched' during the microwaving procedure. The immunohistochemical procedure was repeated again using the microwave antigen retrieval step but with the peroxidase quenching step after the microwaving procedure rather than prior to it. On this occasion there was no evidence of staining. It was then decided to use the pressure cooker technique to try and unmask any of the antigen which might have been present but this time the polyclonal anti-human TNF- α bound to all the sections, as with the microwaving technique, because the endogenous peroxidase activity in the tissue had become 'unquenched'. The immunohistochemical procedure was repeated, again using the pressure cooker antigen retrieval step but with the peroxidase quenching step after the pressure cooking procedure rather than prior to it but on this occasion there was no evidence of staining.

This result was unexpected as the Genzyme polyclonal rabbit anti-human TNF- α had been reported as binding to formalin-fixed paraffin embedded canine patella tissue [Pickvance *et al.*, 1993] so it had been expected to bind to human tissue. At this point it was decided to use frozen sections.

6.1.5.1.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.5.1.1.

6.1.5.2 Frozen Sections

6.1.5.2.1 Positive Control Tissue - Reactive Human Tonsil

Frozen sections taken from reactive human tonsil tissue were titrated against the polyclonal rabbit anti-human TNF- α (1/50, 1/100, 1/200, 1/500 and 1/1000) and there was a little staining visible in the section incubated with the antiserum at the 1/50 dilution. The frozen sections were again titrated against the antibody, this time at different dilutions (1/12.5, 1/25, 1/50 and 1/100). Surprisingly, at these dilutions, the control rabbit serum {Sigma} was binding extremely strongly to the human tonsil tissue. This was specific binding as there was no evidence of staining in the negative control sections (see photo. 6.1.10 {C & D}). (The rabbits that the control serum had come from must have had anti-human antibodies within their circulation (probably against the humans who had looked after them).) Fortunately the polyclonal anti-human TNF- α showed its optimum binding at the 1/25 dilution (see photo. 6.1.10 {A & B}) whereas the control serum displayed optimum binding at the 1/12.5 dilution, so it was conclusive that the antiserum was binding to TNF- α within the tissue. An attempt was made to dilute the antiserum sufficiently so that all equivalent binding of the control rabbit serum would be negligible but unfortunately the antiserum did not bind to the tissue sections satisfactorily at greater dilutions

6.1.5.2.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.5.2.1.

The degree of staining was greater in the patients who were *Helicobacter pylori*-positive, both with (see photo. 6.1.8a) and without gastritis (see photo. 6.1.8b) than those patients who were *Helicobacter pylori*-negative (see photo. 6.1.9). All the samples had some degree of staining within the gastric glands of the antrum. Sections from the patients who were *Helicobacter pylori*-positive had significantly more staining within the lamina propria and this was greater in the samples from patients with gastritis. This was accompanied by an inflammatory response within the tissue as the lamina propria contained many mononuclear cells such as lymphocytes, macrophages and plasma cells. Neutrophils were visible within all the gastric mucosa samples although they were in greater numbers in the samples from patients with gastritis. The lamina propria of the patients who were *Helicobacter pylori*-negative contained very few mononuclear cells. *Helicobacter pylori* were also visible within the gastric pits of the antrum in tissue from patients who were *Helicobacter pylori*-positive. (These bacteria are difficult to visualise in frozen sections which have not been H & E stained, however, one is visible in the FFPE gastric mucosa tissue (see photo. 6.1.14b.))

6.1.6 Genzyme Polyclonal Rabbit Anti-Human IL-1 α (IgG Fraction)

6.1.6.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.6.1.1 Positive Control Tissue - Reactive Human Tonsil

These sections were processed as described in section 6.1.5.1.1 (for the polyclonal rabbit anti-human TNF- α) with a similar lack of success.

This result was unexpected as the Genzyme polyclonal rabbit anti-human IL-1 α had been reported as binding to formalin-fixed paraffin embedded human brain tissue at a 1/1000 dilution [Griffin *et al.*, 1989]. At this point it was decided to use frozen sections.

6.1.6.1.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.6.1.1.

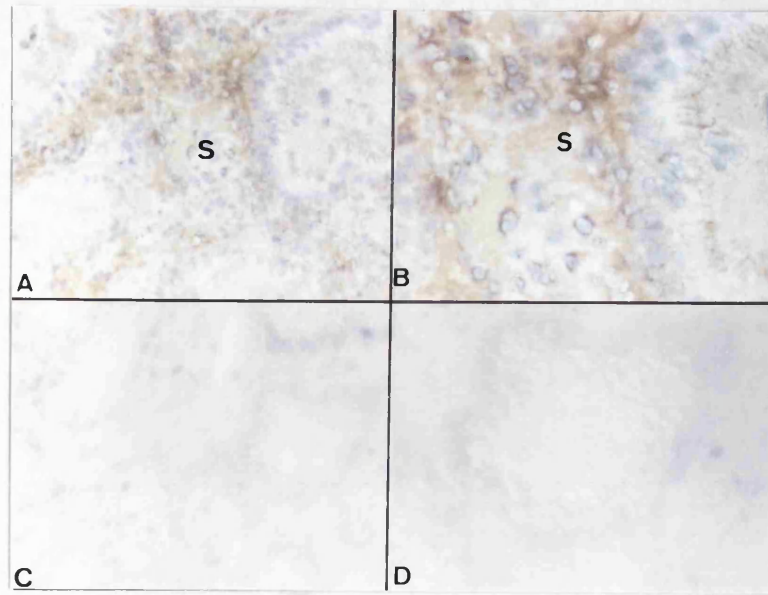


Photo. 6.1.8a

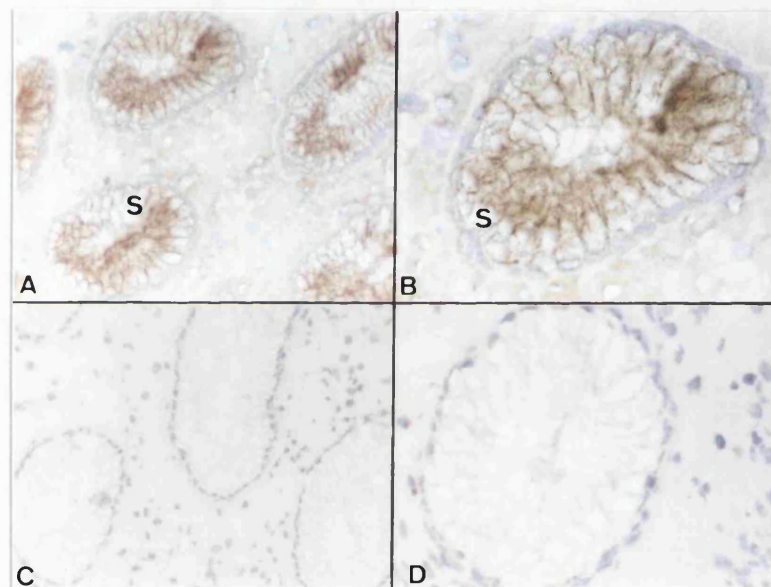


Photo. 6.1.8b

Photo. 6.1.8 Frozen Sections of Human Gastric Mucosa Taken From the Antrum of *Helicobacter pylori*-Positive Patients. Brown Staining (S) Indicates the Presence of TNF- α

a. *Helicobacter pylori*-Positive With Gastritis

A & B = Gastric mucosa incubated with polyclonal anti-human TNF- α . Moderate staining within the lamina propria indicates increased levels of TNF- α when compared with normal control tissue.

C & D = Gastric mucosa incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

b. *Helicobacter pylori*-Positive Without Gastritis

A & B = Gastric mucosa incubated with polyclonal anti-human TNF- α . Light/moderate staining within the lamina propria indicates increased levels of TNF- α when compared with normal control tissue although levels are lower than those observed in patients with gastritis.

C & D = Gastric mucosa incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

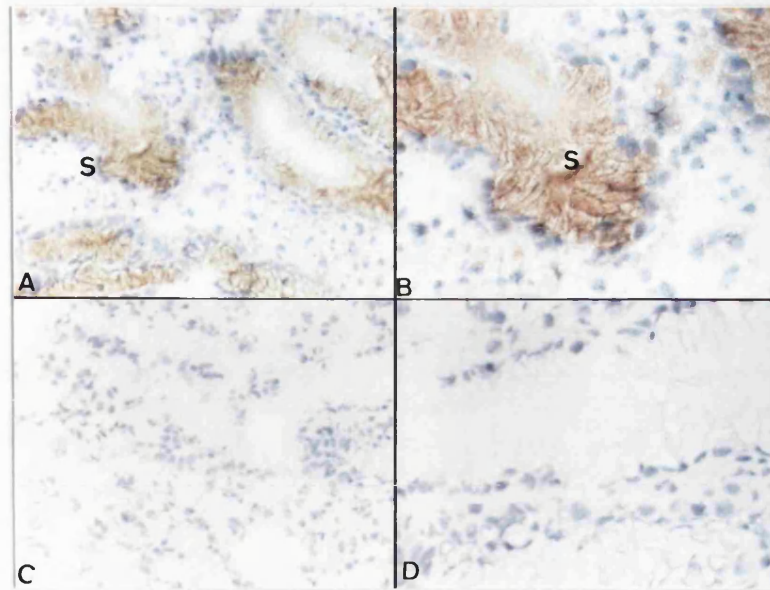


Photo. 6.1.9

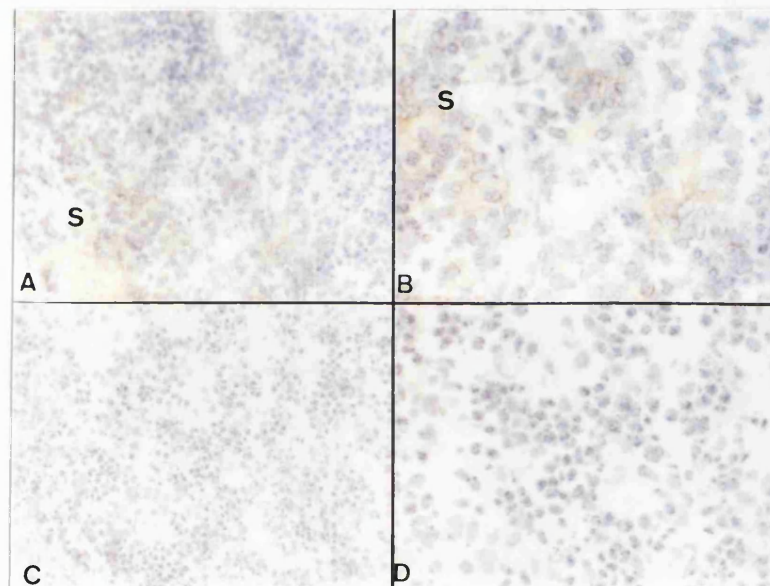


Photo. 6.1.10

Photo. 6.1.9 Frozen Sections of Human Gastric Mucosa Taken From the Antrum of *Helicobacter pylori*-Negative Patients (Normal Control). Brown Staining (S) Indicates the Presence of TNF- α

A & B = Gastric mucosa incubated with polyclonal anti-human TNF- α . Light staining within the lamina propria indicates low levels of TNF- α within normal control tissue.

C & D = Gastric mucosa incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

Photo. 6.1.10 Frozen Sections of Human Reactive Tonsil Used as Positive Control Tissue for the Polyclonal Rabbit Anti-Human TNF- α . Brown Staining (S) Indicates the Presence of TNF- α

A & B = Reactive tonsil incubated with polyclonal anti-human TNF- α . Light/moderate staining indicates that the polyclonal anti-human TNF- α has bound to the TNF- α within the tonsil tissue demonstrating that the antibody has bound successfully {A \times 60 & B \times 120}

C & D = Reactive tonsil incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

6.1.6.2 Frozen Sections

6.1.6.2.1 Positive Control Tissue - Reactive Human Tonsil

Frozen sections taken from reactive human tonsil tissue were titrated against the polyclonal rabbit anti-human IL-1 α (IgG fraction) (1/50, 1/100, 1/200, 1/500 and 1/1000), however, there was no evidence of staining visible. The frozen sections were again titrated against the antibody, this time at different dilutions (1/12.5, 1/25, 1/50 and 1/100). On this occasion there was some staining visible at the 1/12.5 and 1/25 dilutions. Surprisingly, at these dilutions, the control rabbit serum (IgG fraction) {DAKO} was binding as strongly to the human tonsil tissue. This was specific binding as there was no evidence of staining in the negative control sections (see photo. 6.1.13 {C & D}). (The rabbits that the control serum had come from must have had anti-human antibodies within their immune system (probably against the humans who had looked after them).) Unfortunately the control rabbit serum (IgG fraction) {DAKO} and the polyclonal anti-human IL-1- α showed optimum binding at the 1/12.5 dilution (see photo. 6.1.13 {A & B}), so it was inconclusive that the antiserum was binding to IL-1 α within the tissue. This result was surprising as the Genzyme anti-human IL-1 α had been reported as binding to frozen sections of human chorionic villi at a dilution of 1/50 [Haynes *et al.*, 1993].

6.1.6.2.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.6.2.1.

The degree of staining was greater in the patients who were *Helicobacter pylori*-positive, both with (see photo. 6.1.11a) and without gastritis (see photo. 6.1.11b) than those patients who were *Helicobacter pylori*-negative (see photo. 6.1.12). All the samples had some degree of staining within the gastric glands of the antrum. The lamina propria of the patients who were *Helicobacter pylori*-positive with gastritis was full of inflammatory cells, such as lymphocytes, macrophages and plasma cells, and was positively stained for anti-human IL-1 α whereas this response was absent in the samples from patients who were *Helicobacter pylori*-negative. There was very little if any positive staining within the lamina propria of samples taken from patients who were

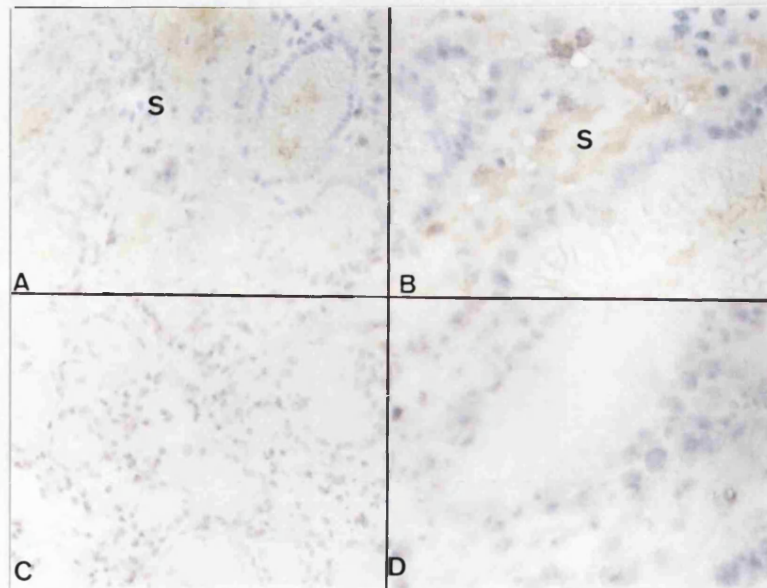


Photo. 6.1.11a

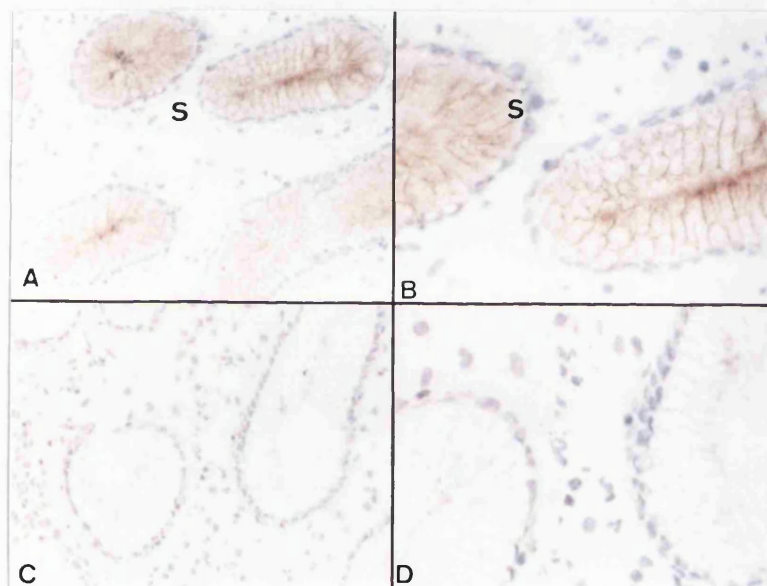


Photo. 6.1.11b

Photo. 6.1.11 Frozen Sections of Human Gastric Mucosa Taken From the Antrum of *Helicobacter pylori*-Positive Patients. Brown Staining (S) Indicates the Presence of IL-1 α

a. *Helicobacter pylori*-Positive With Gastritis

A & B = Gastric mucosa incubated with polyclonal anti-human IL-1 α . Light/moderate staining within the lamina propria indicates increased levels of IL-1 α when compared with normal control tissue.
C & D = Gastric mucosa incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

b. *Helicobacter pylori*-Positive Without Gastritis

A & B = Gastric mucosa incubated with polyclonal anti-human IL-1 α . Light staining within the lamina propria indicates increased levels of IL-1 α when compared with normal control tissue although levels are lower than those observed in patients with gastritis.
C & D = Gastric mucosa incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

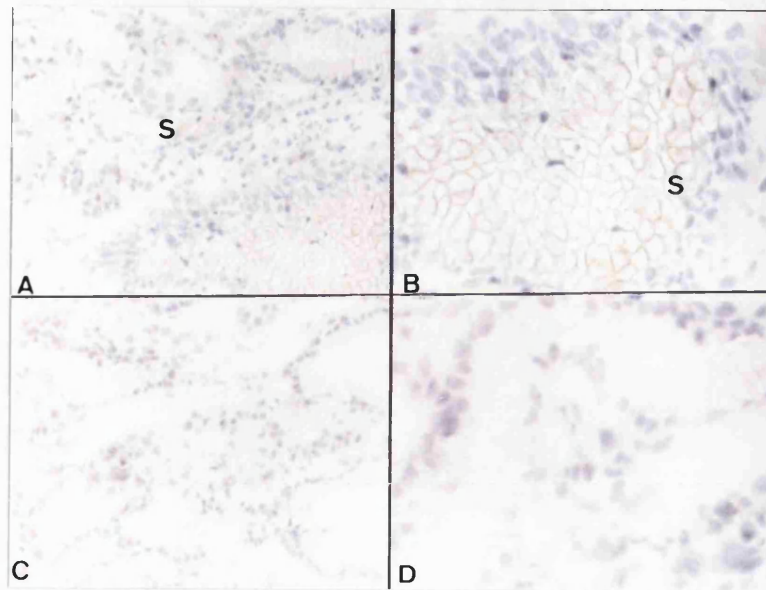


Photo. 6.1.12

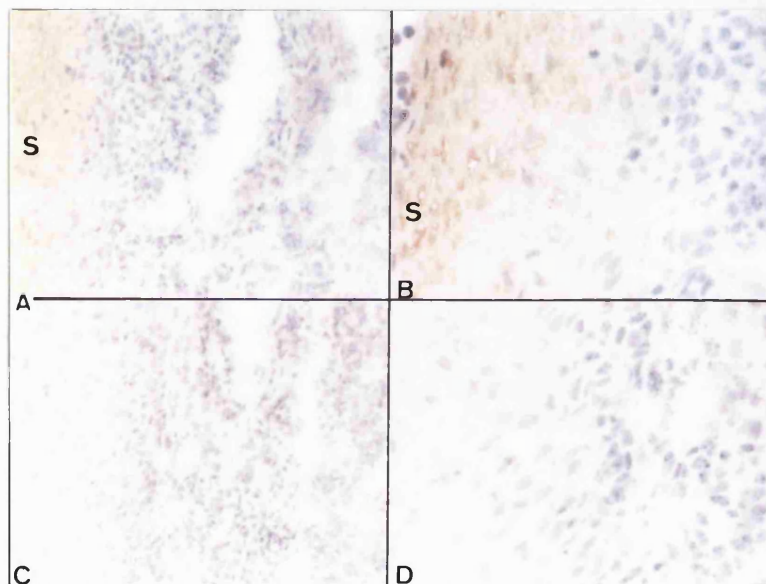


Photo. 6.1.13

Photo. 6.1.12 Frozen Sections of Human Gastric Mucosa Taken From the Antrum of *Helicobacter pylori*-Negative Patients (Normal Control). Brown Staining (S) Indicates the Presence of IL-1 α

A & B = Gastric mucosa incubated with polyclonal anti-human IL-1 α . Slight staining within the lamina propria indicates low levels of IL-1 α within normal control tissue.

C & D = Gastric mucosa incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

Photo. 6.1.13 Frozen Sections of Human Reactive Tonsil Used as Positive Control Tissue for the Polyclonal Rabbit Anti-Human IL-1 α . Brown Staining (S) Indicates the Presence of IL-1 α

A & B = Reactive tonsil incubated with polyclonal anti-human IL-1 α . Light/moderate staining indicates that the polyclonal anti-human IL-1 α has bound to the IL-1 α within the tonsil tissue demonstrating that the antibody has bound successfully {A \times 60 & B \times 120}

C & D = Reactive tonsil incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

Helicobacter pylori-positive without gastritis. *Helicobacter pylori* were visible within the gastric pits of the antrum in samples from patients who were *Helicobacter pylori*-positive. (These bacteria are difficult to visualise in frozen sections which have not been H & E stained, however, one is visible in the FFPE gastric mucosa tissue (see photo. 6.1.14b.))

6.1.7 Anti-Human IL-8

6.1.7.1 Serotec Monoclonal Rabbit Anti-Human IL-8 (IgG Fraction)

6.1.7.1.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.7.1.1.1 Positive Control Tissue - Reactive Human Tonsil

These sections were processed as described in section 6.1.3.1.1 (for the monoclonal hamster anti-mouse IL-1 β) with a similar lack of success. At this point it was decided to use frozen sections.

6.1.7.1.1.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.7.1.1.1.

6.1.7.1.2 Frozen Sections

6.1.7.1.2.1 Positive Control Tissue - Reactive Human Tonsil

Frozen sections taken from reactive human tonsil tissue were titrated against the Serotec monoclonal mouse anti-human IL-8 (IgG fraction) (1/50, 1/100, 1/200, 1/500 and 1/1000), however, there was no evidence of staining visible. The frozen sections were again titrated against the antibody, this time at different dilutions (1/12.5, 1/25, 1/50 and 1/100), again there was no evidence of staining. As a result it was decided to cease work with this antiserum and to purchase a polyclonal anti-human IL-8 as it was thought that this might be more suitable for immunohistochemistry.

6.1.7.1.2.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.7.1.2.1.

6.1.7.2 Genzyme Polyclonal Rabbit Anti-Human IL-8 (IgG Fraction)

6.1.7.2.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.7.2.1.1 Positive Control Tissue - Reactive Human Tonsil

These sections were processed as described in section 6.1.5.1.1 (for the polyclonal rabbit anti-human TNF- α) with a similar lack of success. At this point it was decided to use frozen sections.

6.1.7.2.1.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.7.2.1.1.

6.1.7.2.2 Frozen Sections

6.1.7.2.2.1 Positive Control Tissue - Reactive Human Tonsil

Frozen sections taken from reactive human tonsil tissue were titrated against the polyclonal rabbit anti-human IL-8 (IgG fraction) (1/50, 1/100, 1/200, 1/500 and 1/1000), however, there was no evidence of staining visible. The frozen sections were again titrated against the antibody, this time at different dilutions (1/12.5, 1/25, 1/50 and 1/100), again there was no evidence of staining. As a result it was decided to cease work with this antiserum and to try and find an alternative source of anti-human IL-8, as commercially available antibodies did not appear to be of a high enough standard for immunohistochemistry

6.1.7.2.2.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.7.2.2.1.

6.1.7.3 Donated Monoclonal Mouse Anti-Human IL-8

6.1.7.3.1 Formalin-Fixed Paraffin-Embedded Tissue - RUH, Bath

6.1.7.3.1.1 Positive Control Tissue - Reactive Human Tonsil

This monoclonal antibody had already been used for immunohistochemistry on bowel tissue, at a 1/100 dilution without the trypsin antigen retrieval step, by another research

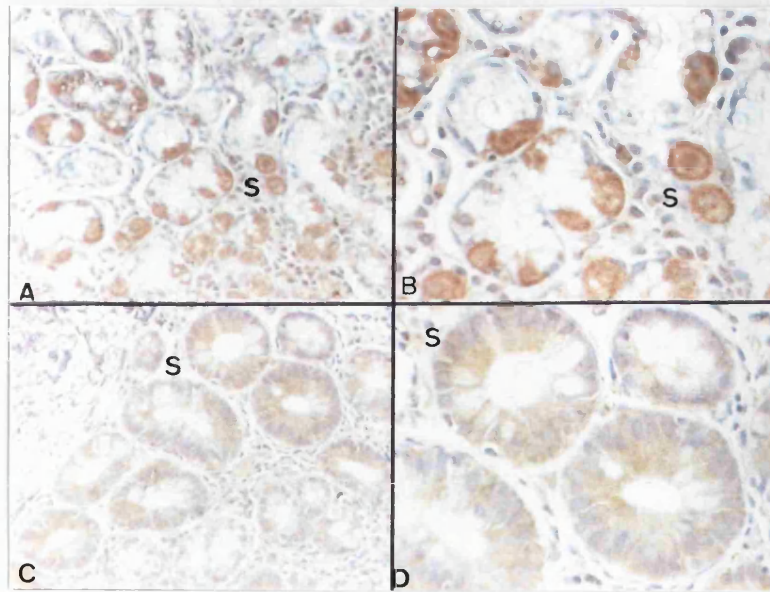


Photo. 6.1.14a

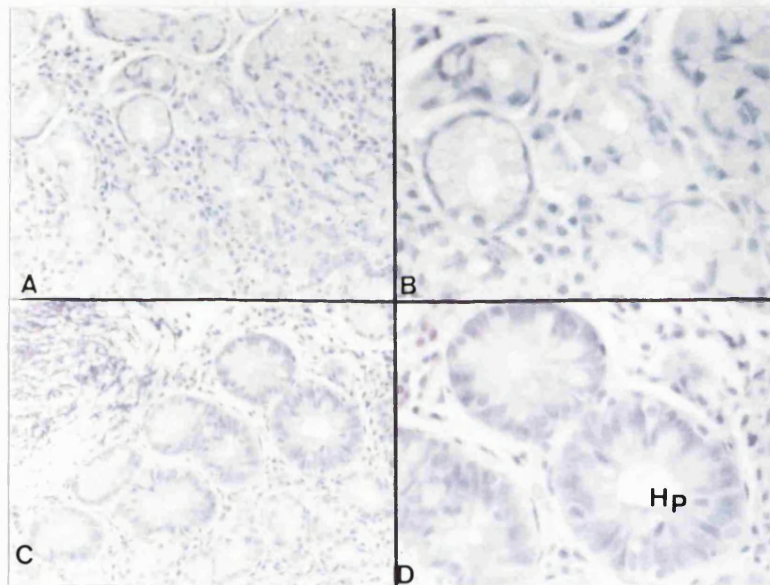


Photo. 6.1.14b

Photo. 6.1.14 FFPE Human Gastric Mucosa Taken From the Antrum of *Helicobacter pylori*-Positive Patient With Gastritis. Brown Staining (S) Indicates the Presence of IL-8

a. Gastric mucosa tissue incubated with monoclonal anti-human IL-8.

A & B = Intense staining within the lamina propria and individual cells within the glandular tissue indicates the presence of high levels of IL-8. Staining within this infected tissue is greater than that observed in similar normal control tissue {A \times 60 & B \times 120}

C & D = Moderate staining within the lamina propria and individual cells lining the gastric pits indicates increased levels of IL-8 when compared with similar normal control tissue {C \times 60 & D \times 120}

b. Gastric mucosa incubated with buffer instead of primary antibody (Negative Control).

A & B = Lack of positive staining indicates absence of any non-specific binding {A \times 60 & B \times 120}

C & D = As above. *Helicobacter pylori* (Hp) bacterium is visible within one of the gastric pits {C \times 60 & D \times 120}

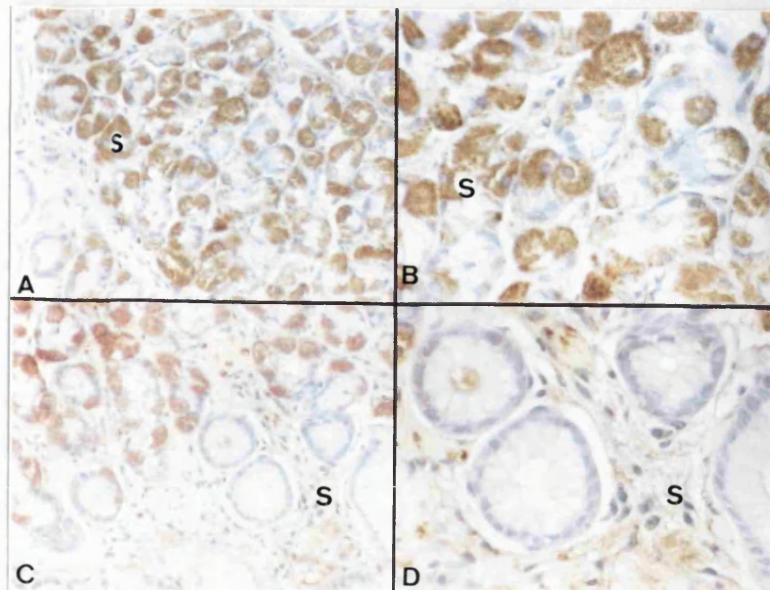


Photo. 6.1.15

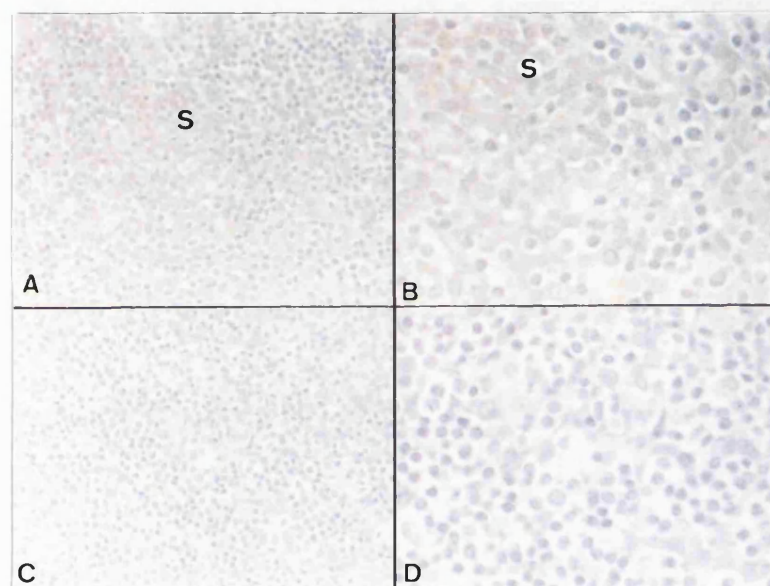


Photo. 6.1.16

Photo. 6.1.15 FFPE Human Gastric Mucosa Taken From the Antrum of *Helicobacter pylori*-Negative Patient (Normal Control) incubated with monoclonal anti-human IL-8. Brown Staining (S) Indicates the Presence of IL-8

A & B = Intense staining within the lamina propria and individual cells within the glandular tissue indicates the presence of high levels of IL-8 within normal control tissue. {A \times 60 & B \times 120}
C & D = Moderate staining within the lamina propria and slight staining within cells lining the gastric pits demonstrates normal levels of IL-8 within this tissue {C \times 60 & D \times 120}

Photo. 6.1.16 FFPE Human Reactive Tonsil Used as Positive Control Tissue for the Monoclonal Anti-Human IL-8. Brown Staining (S) Indicates the Presence of IL-8

A & B = Reactive tonsil incubated with monoclonal anti-human IL-8. Slight staining indicates that the monoclonal anti-human IL-8 has bound to the IL-8 within the tonsil tissue demonstrating that the antibody has bound successfully {A \times 60 & B \times 120}
C & D = Reactive tonsil incubated with buffer instead of primary antibody (Negative Control). Lack of positive staining indicates absence of any non-specific binding to this tissue {C \times 60 & D \times 120}

group working in the same department. The degree of binding obtained was slight but suitable within the positive control tissue (see photo. 6.1.16 {A × B}) and there was no staining visible within the negative control section (see photo. 6.1.16 {C × D}) so all further work was carried out under these experimental conditions.

6.1.7.3.1.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.1.7.3.1.1.

The staining in the tissue sections of samples from both *Helicobacter pylori*-positive patients (see photo. 6.1.14a) and *Helicobacter pylori*-negative patients was very strong (see photo. 6.1.15) which was absent from the negative control sections (see photo. 6.1.14b). In all subjects individual cells within the glandular tissue had a similar degree of binding of the anti-human IL-8. There was also positive staining within the lamina propria of samples from patients who were *Helicobacter pylori*-positive and *Helicobacter pylori*-negative but overall this tended to be more intense in the samples from infected tissue. This was accompanied by an inflammatory response within this tissue as the lamina propria contained many inflammatory cells such as lymphocytes, macrophages and plasma cells. Macrophages and lymphocytes were also visible within the mucosal tissue as were some neutrophils. The lamina propria and the gastric mucosa of the *Helicobacter pylori*-negative patients contained very few if any mononuclear cells. *Helicobacter pylori* were visible, adhering to the inside of the gastric pits of the antrum, in tissue taken from patients who were *Helicobacter pylori*-positive (see photo. 6.1.14b).

6.1.7.3.2 Frozen Sections

6.1.7.3.2.1 Positive Control Tissue - Reactive Human Tonsil

Frozen sections taken from reactive human tonsil tissue were incubated against the donated monoclonal mouse anti-human IL-8 at a dilution of 1/100. However, no staining was visible in the tonsil tissue. Due to lack of time and antibody it was impossible to carry out a titration of the donated anti-human IL-8 on the frozen sections to determine whether or not it would bind to frozen tissue and if so at what concentration.

6.1.7.3.2.2 Experimental Human Gastric Mucosa Tissue

These sections were processed as described above in section 6.3.7.2.2.1.

6.1.8 Protein Blotting

All three of the anti-mouse TNF- α antisera, the polyclonal anti-mouse IL- α and the polyclonal anti-human TNF- α gave a positive result during 'dot blot' analysis. However, the polyclonal anti-human IL-1 α and the monoclonal anti-human IL-4 did not bind to their respective recombinant proteins at any of the concentrations blotted onto the membrane (50, 10, 1, 0.1 and 0.01 μ g). It was not possible to conduct 'dot blot' analysis with the hamster monoclonal anti-mouse IL-1 β as recombinant IL-1 β was unavailable. The donated monoclonal anti-human IL-8 had been reported as recognising the respective human protein [Jordan *et al.*, 1996] and 'dot-blot' analysis had been conducted by another research group within the department. Consequently it was not considered necessary to repeat the experiment for this project.

6.1.9 Neutralisation

The staining intensity was significantly reduced in the sections of positive control tissue which had been incubated with either the polyclonal anti-mouse TNF- α or polyclonal anti-mouse IL-1 α after the buffer containing the respective antiserum had been pre-incubated with the relevant recombinant protein. The maximum staining for both the polyclonal anti-mouse TNF- α and the polyclonal anti-mouse IL-1 α was visible in the control sections which had been incubated with buffer which itself had been pre-incubated in the absence of the relevant recombinant protein. The minimum staining observed for both the polyclonal anti-mouse TNF- α and the polyclonal anti-mouse IL-1 α was in the control tissue sections which had been incubated with buffer containing antiserum which had been incubated previously with 5 ng of the relevant recombinant protein.

6.2 DISCUSSION - ROLE OF CYTOKINES IN ULCER FORMATION

6.2.1 Formalin-Fixed Paraffin-Embedded Tissue - The Bath Clinic, Bath

6.2.1.1 Rat Small Bowel Tissue

Initially the Genzyme polyclonal rabbit anti-mouse IL-1 α was used with sections of formalin-fixed paraffin-embedded tissue which had been processed by The Bath Clinic. (Problems had been encountered in obtaining sections from the RUH.)

The activated lung sections were treated as previously described for sections which had been processed by the RUH. However, there was always a very high level of background staining, which proved impossible to remove at a 1/500 dilution, and this problem was even worse in sections of rat small bowel tissue. Incubation times were altered, as previously described for this antibody, and differing dilutions were also tried to no avail. In addition the level of staining was inconsistent as one dilution which would cause intense staining on some occasions (1/1000) would result in no staining at all during further immunohistochemical procedures, even though it was being used on tissue sections from the same block. Initially it was thought that the problem lay with the antibody, however, it was eventually discovered that the embedding and sectioning procedures had been at fault. This was realised when further experiments using the IgG fraction of the anti-mouse TNF- α on these sections, processed by The Bath Clinic, resulted in similar inconsistencies.

Firstly the tissue had been embedded at a temperature approximately 10 °C higher than that recommended for immunohistochemistry and secondly the sections had been dried at 70 °C for one hour rather than at 37 °C for 24 to 48 hours. As a result the proteins in the sections had become denatured and this was responsible for the high level of background staining found in conjunction with the polyclonal antibodies. Once these mistakes had been realised, more sections were cut from the paraffin blocks and dried at 37 °C for 24 to 48 hours. However, there was still a problem with staining as a 1/1000 dilution of the anti-mouse IL-1 α would give a high degree of staining on some sections but none on others. It was then discovered that if sufficient sections were cut from the block initially (approximately 24) the ensuing sections would respond to the antibody in a

similar manner to sections cut from tissue processed by the RUH, as the proteins in the middle of the tissue block had not been denatured. The results obtained from these rat small bowel sections, cut from blocks processed by The Bath Clinic, then confirmed the results obtained from sections prepared by the RUH.

6.2.1.2 Human Gastric Mucosa Tissue

A similar problem was encountered with sections from blocks of gastric mucosa tissue which had been processed by The Bath Clinic. However, these samples were so small that the proteins were denatured all through the tissue blocks. The monoclonal anti-human IL-8 did bind to tissue sections cut from these blocks, not with the same intensity as that seen in the sections cut from blocks processed by the RUH, but sufficiently to confirm the results obtained with RUH processed tissue.

6.2.2 Commercially Available Antibodies

6.2.2.1 Genzyme Polyclonal Anti-Mouse TNF- α

This antibody had been reported to bind to sections of formalin-fixed paraffin-embedded rat kidney [Diamond & Pesek, 1991]. It did in fact bind to sections taken from activated rat lung, activated rat spleen and ulcerated rat small bowel tissue, with a varying degree of staining, but with only half the intensity of that obtained with the control rat serum at an equivalent protein concentration. The possible explanation for this could be that the standard of the antiserum had deteriorated since the original reported experiments [Diamond & Pesek, 1991] or that it had not been compared with control rat serum, as Diamond & Pesek [1991] did not make any reference to such control serum sections being included during the immunohistochemical procedures.

This antibody did recognise recombinant mouse TNF- α during 'dot blot' analysis but with less than half the response of that seen with the donated anti-mouse TNF- α at the equivalent concentration. Although this was a positive result the antibody was not suitable for immunohistochemistry because the control sera bound with greater intensity. While using the IgG fractions of the donated anti-mouse TNF- α and control rabbit (AH) serum it was noticed that the IgG fraction of the antiserum lost activity after

approximately six months so that the IgG fraction of the control rabbit serum produced staining of twice the intensity when used at the equivalent concentration. (This was in spite of the fact that the two IgG fractions had been processed at the same time and kept in the same freezer at -20°C.) The possibility is that the two Genzyme polyclonal anti-mouse TNF- α antisera had deteriorated in a similar fashion prior to purchase.

6.2.2.2 Genzyme Monoclonal Anti-Mouse IL-1 β and Monoclonal Anti-Mouse IL-4

The lack of results with these two antibodies was very disappointing. It had been suspected that they might not be suitable for use on formalin-fixed paraffin-embedded tissue but initially this was the only type of section available. (The two histopathology laboratories regularly processed formalin-fixed tissue, so it was relatively easy to include samples required for research purposes along with the routine samples, whereas the cutting of frozen sections was considered to be too laborious to be conducted on a regular basis.) It had been hoped that the newer methods of antigen retrieval would result in these two antibodies binding to the formalin-fixed paraffin-embedded tissue but unfortunately they did not. These antibodies had been reported to bind to frozen sections [Khoury *et al.*, 1992; Hancock *et al.*, 1993; Wu *et al.*, 1994], so it was unexpected when they did not.

The negative result obtained during 'dot blot' analysis indicated that even if the anti-human IL-4 had bound to the positive control tissue (human reactive tonsil) it was unlikely to have bound to the rat IL-4 protein despite having been reported in the literature as doing so [Khoury *et al.*, 1992; Hancock *et al.*, 1993]. It was possible that the lack of positive staining with these two monoclonal antibodies may have been because the respective antigens had been destroyed during the endogenous peroxidase quenching step as this is not recommended for frozen sections [Boenisch, 1989]. Usually an alkaline phosphatase staining procedure is used on frozen sections [Boenisch, 1989] as this does not require the endogenous peroxidase activity to be quenched, thus the hydrogen peroxide/methanol step can be omitted. Unfortunately the reagents necessary for this procedure were not available.

6.2.2.3 Genzyme Polyclonal Anti-Human TNF- α and Polyclonal Anti-Human IL-1 α

These two antibodies were similarly disappointing as both the anti-human TNF- α and the anti-human IL-1 α had been reported as being suitable for use on formalin-fixed paraffin embedded tissue in immunohistochemistry [Griffin *et al.*, 1989; Pickvance *et al.*, 1993]. Although the anti-human TNF- α did bind faintly to frozen sections at a 1/50 dilution the control serum bound to a similar extent and it was only at the 1/25 dilution that the staining intensity with the antiserum was more (two-fold) than that seen with the control serum. Similarly the anti-human IL-1 α did bind to frozen sections of gastric mucosa tissue at a dilution of 1/12.5 but always the staining observed was of equal intensity to that obtained with the control rabbit serum.

During routine immunohistochemical procedures most antibodies are used at dilutions of 1/400-1/500 so the fact that these antibodies had to be used at such a high concentration was indicative of how poor they were. The minimum volume of buffer required to cover a small section is 100 μ l which meant that when using the anti-human TNF- α a minimum of 4 μ l per section had to be used whereas when using the anti-human IL-1 α the minimum was 8 μ l per section. Consequently these antibodies were used up very quickly which made them extremely expensive to use.

The positive result obtained with the anti-human TNF- α during the 'dot blot' analysis confirmed that this antibody was binding to TNF- α present in the gastric mucosa samples. However, the negative result with the anti-human IL-1 α would appear to suggest that this antiserum (IgG fraction) was not binding specifically to the IL-1 α protein but to other antigens within the tissue, which were also recognised by the naturally occurring antibodies within the IgG fraction of the control rabbit serum. The Genzyme polyclonal anti-human IL-1 α had been reported by Genzyme as binding to frozen sections of human tissue at a 1/50 dilution [Haynes *et al.*, 1993]. This antibody had in fact been used at that concentration but on reading the literature [Haynes *et al.*, 1993] it would appear that no trace of IL-1 α was found in the tissue they were investigating. This research group had compared the binding of the antiserum with control rabbit serum but had not apparently included a positive control tissue in with

their experimental procedure. As a result it was impossible to say for certain that IL-1 α was not present in their experimental tissue. The positive staining obtained when using the anti-human IL-1 α on the gastric mucosa samples did appear to indicate that this antiserum may be binding to IL-1 α and the presence of TNF- α within the same tissue would seem to suggest that IL-1 α could be present. It would appear, however, that this polyclonal anti-human IL-1 α was never suitable for immunohistochemistry.

6.2.3 Comparison of Staining Intensity in Rat Small Bowel Tissue

6.2.3.1 Donated Polyclonal Rabbit Anti- Mouse TNF- α (IgG Fraction)

The staining in tissue sections, taken from animals with ulcers which had been dosed with indomethacin ID₆₀ acute, was mostly very slight. The only area of these tissue sections where the anti-TNF- α bound strongly was in the area of the Peyer's patches associated with an ulcer or an erosion. The unulcerated tissue immediately surrounding the ulcer and the unulcerated areas of small bowel between ulcers were all stained very slightly when compared with the rest of the experimental small bowel tissue. The positive staining for TNF- α was observed in mononuclear cells within the lymphoid follicle of the activated Peyer's patches. Tissue sections taken from the rats which had been given an acute dose of indomethacin ID₃₀ had approximately double the staining seen in the unulcerated areas of the indomethacin ID₆₀ animals as did the sections taken from the animals which had received the vehicle (10 per cent (v/v) Tween-80) although the staining was slightly greater in the latter. The small bowel samples which had the highest degree of staining throughout the whole tissue section came from the control animals (see table 6.1.1.1.3).

TNF- α is the pivotal cytokine involved in the inflammatory process and from these results it would appear that there are high levels of the cytokine present in the normal small bowel tissue of rats. The animals with ulcers had very little TNF- α in the unulcerated areas of small bowel which would suggest that the normal TNF- α 'pool' had been depleted. In the animals treated with indomethacin ID₃₀, where ulcers had not formed, the inflammatory response had probably already begun, resulting in a lowering of

the normal TNF- α 'pool' but not to the extent of that seen in the indomethacin ID₆₀ animals.

6.2.3.2 Genzyme Polyclonal Rabbit Anti- Mouse IL-1 α in Hyperimmune Serum

The staining in tissue sections containing ulcers, taken from animals which had been dosed with indomethacin ID₆₀ acute, was very slight throughout the entire tissue section although in some unulcerated areas the degree of staining was slightly greater. Staining with the anti-mouse IL-1 α , however, was greatest in the animals given indomethacin ID₃₀ and the antiserum bound with equal intensity all through the small bowel tissue. The majority of the positive staining was visible within individual cells of the intestinal epithelium covering the villi tips although there was also some positive staining within the lamina propria which contained many mononuclear cells associated with an inflammatory response. The staining in the sections cut from tissue which had come from the control and vehicle control animals was of equal intensity and was approximately twice that seen in the ulcerated tissue but half that visible in tissue from animals dosed with indomethacin ID₃₀. The positive staining in the control tissues was mostly confined to individual cells of the epithelium covering the villi, as the lamina propria contained few mononuclear cells in comparison with the other tissues. These results appear to show that normal rat small bowel tissue has high levels of IL-1 α . The higher levels of IL-1 α seen in the tissue from rats dosed with indomethacin ID₃₀, would seem to suggest that the inflammatory response was at a maximum as there were many inflammatory cells within the lamina propria and twice the number of intestinal epithelial cells than normal were positively stained. Thus it would appear that there was an initial inflammatory cytokine response, prior to ulcer formation, as a result of indomethacin ingestion. The low levels of IL-1 α in the small bowel tissue from the animals with the ulcers would appear to indicate that the initial inflammatory response has diminished and the normal 'pool' of IL-1 α has been depleted. This could be due to the production of IL-1 receptor antagonist (IL-1ra) and IL-4 which down regulate the cellular immune response to infection by inhibiting many of the leucocyte responses [Danzer *et al.*, 1994; D'Andrea *et al.*, 1995].

6.2.4 Presence of Cytokines in Rat Small Bowel Tissue

Other researchers have reported that IL-1 is abundant in epithelial lining cells [Guenard *et al.*, 1991] and the relatively high levels of TNF- α and IL-1 α within the normal small bowel tissue of the rat would seem to indicate that the gastrointestinal epithelium is usually in an 'inflammatory state', probably as a direct result of the normal bacteria within the lumen.

The structure and function of the gastrointestinal tract is dependent upon the presence of resident bacterial flora [Abrams *et al.*, 1963; Thompson & Trexler, 1971; Coates & Fuller, 1977; Simon & Gorbach, 1986, 1987] and studies in germ-free rats reveal that in these animals the intestinal wall is significantly thinner and less cellular. The villi also are thinner and more pointed, the crypts are shallower and the total mucosal surface is much reduced [Gordon & Bruchorer-Kardoss, 1961; Heneghan, 1984]. In normal rats the mucosal cells are columnar whereas in germ-free rats they are cuboidal and uniform in both size and shape. The lack of bacteria also results in a lamina propria which consists of a sparse stroma infiltrated with only a few macrophages and lymphocytes. As there is little to stimulate an immunological reaction, plasma cells are absent and Peyer's patches are smaller with fewer germinal centres. Cellular turnover is also decreased, with the mucosal cells taking twice as long to migrate from crypt to villus as those in conventional rats [Abrams *et al.*, 1963; Leshner *et al.*, 1964]. Once germ-free rats have been colonised by the normal rat gastrointestinal microflora, the small and large bowel rapidly assumes a conventional appearance [Abrams *et al.*, 1963; Kenworthy, 1971]. The lamina propria takes on its customary mien and becomes infiltrated by lymphocytes, macrophages and plasma cells. Thus, what is termed normal morphology is suggestive of a chronic inflammatory response [Simon & Gorbach, 1986, 1987]. This would appear to provide additional evidence that the gastrointestinal epithelium is constantly under attack from luminal bacteria, their toxins and by products, and explains the relatively high level of cytokine activity seen when compared to other areas of the body. The simultaneous production of prostaglandins probably attenuates this 'chronic inflammatory response' maintaining a delicate balance.

6.2.5 Possible Role of NSAIDs

The delicate balance between the proinflammatory and antiinflammatory processes within the gastrointestinal epithelium is disturbed by the administration of NSAIDs, particularly the more acidic ones such as indomethacin and diclofenac. The initial topical damage is probably due to their ability to enter into the cells of the epithelium lining the gastrointestinal tract where they alter mitochondrial respiration by uncoupling oxidative phosphorylation [Brody, 1956; Penniall, 1958; Packer *et al.*, 1959; Jeffrey & Smith, 1959; Whitehouse, 1964; Smith & Dawkins, 1971; Kawai *et al.*, 1985]. This initiates a fall in the production of ATP which is necessary for maintaining the tight junctions between the epithelial cells of the gastrointestinal tract [Meza *et al.*, 1980; Madara & Dharmasathaphorn, 1985; Madara *et al.*, 1986, 1987] and probably is the cause of the small bowel permeability, observed within six hours of NSAID administration [Bjarnason *et al.*, 1992a]. ATP is also required for the transfer of many molecules across membranes in order to maintain the ion balance both within the intracellular compartment and between it and the extracellular space [Harrison & Lunt, 1980]. Failure to maintain this ion balance can lead to membrane disruption as the cell swells and bursts resulting in the intracellular contents being exposed to the outside milieu. The protection afforded by concomitant administration of prostaglandin E₂ and its analogues [Robert, 1974, 1975, 1979; Fang *et al.*, 1977; Tabata & Okabe, 1983; Romain *et al.*, 1987] is probably partly mediated through this process as these compounds help maintain the ion balance within the intracellular compartment and as a result the integrity of the cell membrane is maintained [Miller & Jacobson, 1979].

Once the intestinal barrier has been compromised, bile, bacterial toxins and other noxious agents are able to pass through into the gastrointestinal epithelium. This probably causes an inflammatory response as bile salts are able to induce IL-1 synthesis [Dinarello, 1991] and bacterial endotoxins and exotoxins stimulate leucocytes to secrete TNF- α [Dinarello, 1991]. This initiates the inflammatory cytokine cascade [Fong *et al.*, 1989; Tracey & Cerami, 1993] by stimulating the surrounding epithelial cells and leucocytes to secrete IL-1 α in response to the TNF- α and this may explain the increased production of IL-1 α observed within the intestinal epithelial cells of animals dosed with indomethacin ID₃₀.

The decreased staining for TNF- α observed within the indomethacin ID₃₀ and ID₆₀ tissues is probably due to cellular responses to TNF- α .

In experimental animals injected with LPS, a TNF- α peak is visible at 1.5 hours [Tracey & Cerami, 1993] but after 3-4 hours the amount of TNF- α within the serum is very small [Zuckerman *et al.*, 1991]. The serum half-life of TNF- α and other cytokines in humans and laboratory animals is short (6 to 20 min) [Tracey & Cerami, 1993] and the rapid decline of circulating TNF- α after peak levels is probably due to the numerous receptors for this cytokine on other cells [Tracey & Cerami, 1993]. A second injection of LPS, 20 hours later, does not elicit a second peak as once mononuclear cells have been exposed to LPS they do not immediately release TNF- α on subsequent exposure [Zuckerman *et al.*, 1991]. This mechanism of endotoxin tolerance lasts for three to four days and after that time is reversible [Williams *et al.*, 1983; Madonna & Vogel, 1985; Madonna *et al.*, 1986; Haslberger *et al.*, 1988]. Thus tolerance to LPS and related substances was probably why there was very little TNF- α within the sections of small bowel tissue from animals dosed with indomethacin ID₃₀ and ID₆₀ as the resident mononuclear cells within the small bowel tissue would have been exposed to TNF- α during the initial inflammatory response, hours before the tissue was excised. The maximal levels for TNF- α within the experimental tissue probably occurred within six hours of drug administration. The intense staining visible within areas of the Peyer's patches, associated with ulcers and erosions, were probably sites of newly recruited leucocytes to the region of inflammation, as the lymphoid follicles are readily accessible to inflammatory mononuclear cells, such as macrophages, lymphocytes and mast cells. These mononuclear cells were probably secreting TNF- α in response to coming into contact either with IL-1 or with the bacteria and/or their toxins diffusing out of the area of necrosis. The kinetics of IL-1 production are different, however, to those of TNF- α . After the initial injection of LPS there is a broad peak at 3-4 hours which continues for 2-3 hours before returning to basal rates at 20 hours post LPS [Zuckerman *et al.*, 1991]. Following a second injection of LPS there is a corresponding second peak which is often greater in magnitude than the peak obtained initially [Zuckerman *et al.*, 1991]. Thus the elevated levels of IL-1 α within the tissue from animals dosed with indomethacin ID₃₀ are probably either due to a protracted response to the original TNF- α exposure, IL-1 can

stimulate its own production and release [Dinarelli *et al.*, 1987; Warner *et al.*, 1987a, 1987b; Schindler *et al.*, 1990], or the result of additional exposure to TNF- α from newly recruited leucocytes. The decrease in staining for IL-1 α in the ulcerated tissue is probably due to the production of both IL-1 receptor antagonist (IL-1ra) and IL-4 which between them down regulate the cellular immune response to infection by inhibiting many of the leucocyte responses [Danzer *et al.*, 1994; D'Andrea *et al.*, 1995]. IL-4 is believed to mediate the balance between the production of IL-1 and IL-1ra by increasing the formation of IL-1ra [Kam *et al.*, 1995] as well by as decreasing the production of IL-1 itself [Hart *et al.*, 1989; Vannier *et al.*, 1992]. Formation of IL-1ra follows shortly after production of IL-1 [Arend *et al.*, 1991; Andersson *et al.*, 1992] and is thought to protect the host from the catastrophic synergistic effects of TNF- α and IL-1 which can be lethal [Waage & Espevik, 1988; Fong *et al.*, 1989; Waage *et al.*, 1989; Munoz *et al.*, 1991; Cannon *et al.*, 1992]. Thus the ability of an animal to become tolerant to bacteria and their toxins is essential if the host is to survive and elicit a controlled inflammatory response [Zuckerman *et al.*, 1991; Andersson, *et al.*, 1992]. (It would have been interesting to see how much IL-4 activity there was within the rat small bowel tissue but unfortunately the anti IL-4 purchased for this purpose was unsuitable.)

There was evidence of increased neutrophil activity within the small bowel tissue from animals dosed with indomethacin ID₆₀, especially surrounding the area of necrosis associated with the ulcer. The primary chemoattractant for neutrophils in rats which have been exposed to bacterial LPS is cytokine-induced neutrophil chemoattractant (CINC) [Rinaldo *et al.*, 1984; Christman *et al.*, 1985, 1989; Watanabe *et al.*, 1991; Iida *et al.*, 1992]. CINC is produced and secreted by epithelial cells [Watanabe & Nakagawa, 1987, 1990; Watanabe *et al.*, 1989a, 1989b, 1993] after activation either by TNF- α or IL-1 [Watanabe *et al.*, 1989b; Nakagawa *et al.*, 1993; Zagorski & De Larco, 1993; Wittwer *et al.*, 1993] in a parallel fashion to IL-8 generation in humans.

The influx of neutrophils into the area of bacterial invasion results in serious damage to the surrounding tissue as the toxins used to defend the host against the invading bacteria also damage the intestinal epithelial cells in the immediate vicinity of degranulation. All cells possess a number of antioxidant enzymes and scavengers to protect themselves

from these injurious agents but often the production of reactive oxygen metabolites overwhelms these defences [Grisham & Granger, 1988]. This inability of the neutrophil to differentiate between foreign and host antigens [Weiss, 1989] thus results in necrosis of the surrounding tissue and this is probably instrumental in the formation of the ulcer. Epithelial cell death is in itself extremely inflammatory as many of the cell products, released into the extracellular space when a cell is destroyed, are themselves exceedingly chemoattractant for neutrophils [Kuby, 1997]. NSAID-induced damage to the gastrointestinal tract is significantly reduced in animals which have been depleted of neutrophils either by methotrexate or anti-neutrophil serum prior to NSAID administration [Wallace *et al.*, 1990, 1991; Lee *et al.*, 1992]. Similarly prevention of neutrophil adherence to the endothelium by an antiserum directed against the neutrophil adhesion protein CD18 also reduces indomethacin-induced ulceration [Vedder *et al.*, 1988; Wallace *et al.*, 1991]. These findings would appear to give support to the proposal that neutrophils are the major culprits responsible for indomethacin-induced injury in rats [Del Soldato *et al.*, 1985; Pihan *et al.*, 1987; Vaananan *et al.*, 1991].

Neutrophils are also attracted to sites of infection by N-formylmethionylleucylphenylalanine (FMLP) [Carp, 1982; Tauber & Babior, 1985], a tripeptide which is released by *Escherichia coli* during replication. *Escherichia coli* are known to have a role in ulcer formation [Robert & Asano, 1977] so once the ulcer has become established and the lesion is full of replicating bacteria the amount of FMLP diffusing out of the necrotic tissue is significant enough to induce an additional neutrophilic response. This probably accounted for the high numbers of neutrophils observed on the periphery of the ulcer in the ulcerated rat small bowel tissue. Although FMLP is nontoxic to mammalian cells it not only attracts neutrophils but also activates them to produce oxygen free radicals, hydrogen peroxide and hypochlorous acid [Tauber & Babior, 1985; Klebanoff, 1992]. These reactive oxygen metabolites are extremely cytotoxic to cells in the surrounding tissue, as well as being lethal to bacteria, and minute amounts of FMLP are known to cause mucosal injury within the small bowel as a direct result of neutrophil activation [Granger *et al.*, 1987; Von Ritter *et al.*, 1988].

In humans neutrophils are also attracted to sites of inflammation by leukotrienes, particularly leukotriene B₄ (LTB₄) [Goetzl & Pickett, 1980; Bray *et al.*, 1981; Palmblad *et al.*, 1981; Grisham & Granger, 1988], however, these agents are not such specific neutrophil chemoattractants as the chemokines, CINC and IL-8, and are able to attract other mononuclear cells to the region of inflammation. It has been reported that LTB₄ is not chemotactic for rat neutrophils [Kreisle *et al.*, 1985; Sugawara *et al.*, 1995], nevertheless it does stimulate the expression of the CD11/CD18 leucocyte adhesion glycoprotein [Zimmerman & McIntyre, 1988; Harlan & Liu, 1992] which enhances neutrophil adherence [Wallace *et al.*, 1991]. Thus the additional neutrophils observed within the experimental rat small bowel tissue are unlikely to be there in response to this inflammatory mediator. Mononuclear cells which are attracted to LTB₄, however, probably release neutrophilic chemoattractants themselves once they have migrated to the area of inflammation and LTB₄ itself stimulates the synthesis of IL-1 [Hwang, 1989].

Initially the up-regulation of LTB₄ synthesis would have occurred as a direct result of cyclooxygenase (COX-1 and COX-2) inhibition by indomethacin. As a consequence 5-lipoxygenase, the first dedicated enzyme in leukotriene synthesis, channels the surplus arachidonic acid down this pathway [Rainsford, 1987; Bjarnason *et al.*, 1989]. This increase in inflammatory leukotrienes is accompanied by the loss of prostaglandin mediated antiinflammatory properties which include prostaglandin inhibition of free radical production and enzyme release by neutrophils [Gryglewski *et al.*, 1987; Kainoh *et al.*, 1990] plus prostaglandin stabilisation of mucosal mast cells [Bickel & Kauffman, 1981; McQueen *et al.*, 1983] and lysosomal membranes [Ferguson *et al.*, 1973]. Mast cells release preformed cytokines [Galli, 1993; Kubes & Granger, 1996] such as TNF- α which is stored prepackaged in granules, readily available for release [Gordon & Galli, 1991, 1994].

An additional stimulus for leukotriene synthesis occurs once the inflammatory cytokine cascade has been initiated as IL-1 also stimulates the production of inflammatory eicosanoids [Cominelli *et al.*, 1989] such as PGE₂ and LTB₄. Evidence supporting this comes from the fact that the increase in hypothalamic PGE₂, observed in fever, is caused by IL-1 mediated release of arachidonic acid via the hydrolysis of phospholipid from cell

membranes [Dinarello, 1991,1994]; and protection against colitis and hyperoxia is partly mediated by IL-1 stimulated prostaglandins [Vannier *et al.*, 1989; White & Ghezzi, 1989]. TNF- α is also capable of stimulating the formation of arachidonic acid [Dinarello, 1994] so an inflammatory response, in the presence of NSAIDs which are inhibitors of cyclooxygenase (COX-1 and COX-2), results in most of the arachidonic acid being channelled into the production of even more inflammatory leukotrienes [Asako *et al.*, 1992; Hudson *et al.*, 1993]. This increase in inflammatory leukotrienes is particularly significant when the increased manufacture of arachidonic acid occurs after ingestion of an NSAID such as indomethacin which predominantly inhibits the COX-1 enzyme [Meade, *et al.*, 1993; Mitchell *et al.*, 1993]. COX-1 is the constitutive form of the enzyme and is responsible for the physiological synthesis of prostaglandins in the gut, kidney, blood platelets [Funk *et al.*, 1991] and other tissues [Simmons *et al.*, 1991]. It is inhibition of this enzyme by NSAIDs which is believed to be responsible for the damage caused to the gastrointestinal epithelium as prostaglandins enhance cellular resistance to injury [Tarnawski *et al.*, 1988]. NSAIDs such as nabumetone, however, selectively inhibit the COX-2 enzyme [Meade, *et al.*, 1993] which is the inducible form responsible for inflammatory prostaglandin synthesis within mononuclear cells such as neutrophils [Kuby, 1997], mast cells [Kuby, 1997], macrophages and monocytes [Aderem *et al.*, 1986; Fu *et al.*, 1990; Hla & Neilson, 1992; O' Sullivan, 1992a, 1992b]. Thus the constitutive form of cyclooxygenase, COX-1, is only partially inhibited after nabumetone ingestion enabling it to fulfil its cellular 'housekeeping' functions [Meade *et al.*, 1993] relatively unhindered. Consequently nabumetone causes very little damage if any to the gastrointestinal epithelium.

In addition to its role of being the primary initiator of the inflammatory cytokine cascade, TNF- α has many other inflammatory properties. TNF- α induces the synthesis and expression of the intercellular adhesion molecules ICAM-1 and ELAM-1 on endothelial cells [Pohlman *et al.*, 1986] and LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) on neutrophils [Dustin & Springer, 1989] which are potent stimuli for neutrophil adherence. Consequently administration of TNF- α to rats results in vasocongestion, leucocyte margination and tissue damage, predominantly within the gastrointestinal tract [Tracey *et al.*, 1986; Remick *et al.*, 1987; Sayers *et al.*, 1988; Sartor *et al.*, 1994] which manifests

itself as a spotty necrosis on the surface of the intestinal epithelium [Remick *et al.*, 1987]. It has been proposed that some of this damage may be due to TNF- α exerting a direct cytotoxic effect on the cells of the gastrointestinal epithelium [Santucci *et al.*, 1995]. Evidence supporting this proposal is provided by the observation that isolated gastric chief cells express TNF receptors and that exposure to TNF causes a dose-dependent necrosis that is prevented by pretreatment with specific anti-TNF receptor monoclonal antibodies [Fiorucci *et al.*, 1993]. An excess of TNF- α would also appear to increase small bowel permeability, as in cultured monolayers of a pig kidney epithelial cell line, TNF- α decreases the resistance in epithelial tight junctions leading to leakage of solutes across electrolyte-secreting epithelial cells [Mullin & Snock, 1990]. Thus high levels of TNF- α within the small bowel, as a consequence of inflammation, would in itself appear to mediate ulcer formation.

6.2.6 Role of Cytokines in Human Gastric Mucosa Tissue

The degree of positive staining obtained for the three antisera used, anti-human TNF- α , anti-human IL-1 α and anti-human IL-8, was not significantly different within the epithelial cells surrounding the antral gastric pits of gastric mucosa samples taken from patients who were either *Helicobacter pylori*-positive or *Helicobacter pylori*-negative. There was an increase in positive staining within the lamina propria, however, which was full of inflammatory mononuclear cells in the samples taken from patients who were *Helicobacter pylori*-positive. The positive staining observed within the epithelial cells of gastric mucosa samples from patients who were *Helicobacter pylori*-negative would seem to suggest that, as with the control rat small bowel samples, normal gastrointestinal epithelium is home to considerable cytokine activity. A similar observation has been made, in respect of gastric mucosa, by other researchers [Ishihara *et al.*, 1996] and is probably the result of exposure to antigens which are ingested into the stomach as a matter of course. There was, however, increased cytokine activity within the gastric mucosa samples which were infected with the bacterium *Helicobacter pylori*. This was probably a result of an inflammatory response to the various toxins of *Helicobacter pylori* [Craig *et al.*, 1992; Neilsen & Andersen, 1992] which include the bacterium's urease [Mai *et al.*, 1992; Harris *et al.*, 1996], lipopolysaccharide [Neilsen *et al.*, 1994], the water soluble *Helicobacter pylori* neutrophil activating protein (HP-NAP) [Evans *et*

al., 1994; 1995] and another, as yet unidentified, protein which is thought to be coexpressed with the product of the *cagA* gene [Akopyants *et al.*, 1995; Lange *et al.*, 1995]. This bacterium and its associated toxins are chemoattractant for monocytes/macrophages [Craig *et al.*, 1992] which are known to secrete IL-1 β and TNF- α (and probably IL-1 α) in response to contact with these toxins [Crabtree *et al.*, 1991; Mai *et al.*, 1991; Moss *et al.*, 1994; Noach *et al.*, 1994; Peek *et al.*, 1995] and the *Helicobacter pylori* urease protein has been found within macrophages inside the lamina propria of biopsy specimens from patients with *Helicobacter pylori* gastritis [Mai *et al.*, 1991]. Thus the inflammatory cytokine cascade, as previously described, is initiated within the gastric mucosa of infected patients.

There was also evidence of increased neutrophil activity within the gastric mucosa of the *Helicobacter pylori*-positive patients especially those with gastritis. Recruitment of neutrophils to the region of inflammation probably occurred as a result of the release of IL-8 from gastric epithelial cells [Crabtree *et al.*, 1994 1995; Crowe *et al.*, 1995; Huang *et al.*, 1995; Sharma *et al.*, 1995], macrophages [Walz *et al.*, 1987; Yoshimura *et al.*, 1987b], T cells [Gregory *et al.*, 1988] and neutrophils [Strieter *et al.*, 1990] which had been stimulated by IL-1 α and TNF- α [Yashimoto *et al.*, 1992; van Damme, 1994]. IL-8 is a potent chemoattractant for neutrophils, directing their migration [Schröder *et al.*, 1987; Yoshimura *et al.*, 1987a, van Damme *et al.*, 1988] to sites of infection and inflammation, although to a lesser extent IL-8 also attracts T cells [Larsen *et al.*, 1989b] and basophils [White *et al.*, 1989]. IL-8 is essential for the invasion of neutrophils across endothelial cell monolayers and synthetic blood-vessel walls [Huber *et al.*, 1991; Smith *et al.*, 1991; Kuijpers *et al.*, 1992], which suggests that it is the mediator prerequisite for neutrophils leaving the systemic circulation in order to reach the area of inflammation. IL-8 also induces neutrophil degranulation at the site of infection which results in the release of lysosomal enzymes such as myeloperoxidase, α -mannosidase, β -glucuronidase, elastase, gelatinase B, vitamin B₁₂-binding protein and lactoferrin [Schröder *et al.*, 1987; Peveri *et al.*, 1988; Willems *et al.*, 1989; Masure *et al.*, 1991]. This particular inflammatory response is believed to have a role in the gastritis associated with *Helicobacter pylori* as levels of IL-8 are markedly increased in areas with chronic gastritis [Crabtree *et al.*, 1993, 1994]. This probably results in an influx of neutrophils

into the infected area and their subsequent adhesion and degranulation results in the neutrophil mediated damage previously described. This is a chronic inflammatory response, however, unlike the acute inflammatory response obtained with an ulcerogenic dose of indomethacin which is heightened by indomethacin's inhibition of cyclooxygenase (COX-1 and COX-2). In most instances *Helicobacter pylori* infection does not automatically result in the formation of a gastric or duodenal ulcer [Sipponen *et al.*, 1989], however, patients taking NSAIDs are more at risk of developing one [Jones *et al.*, 1991].

Helicobacter-pylori positive patients with duodenal ulcer have been found to have higher fasting serum gastrin levels than those patients who only have gastritis [Haruma *et al.*, 1995] and it has been suggested that this hypergastrinaemia is caused by the bacterium's urease activity. The modification of the gastric surface pH via neutralisation of gastric acid by ammonia [Levi *et al.*, 1989b] is believed to result in a lower antral somatostatin concentration [Kaneko *et al.*, 1992]. An alternative explanation for the cause of hypergastrinaemia, however, is that it may be a direct result of the local immunological inflammatory response to the bacterium by the host's innate immune system [Graham *et al.*, 1993]. Samples of gastric mucosa taken from *Helicobacter pylori*-positive subjects, infected with the type 1 strain, have been shown to have elevated levels of the major proinflammatory cytokines IL-1 β and TNF- α as well as IL-6, IL-7, IL-8 and IL-10 [Crabtree *et al.*, 1991; Noach *et al.*, 1994; Peek *et al.*, 1995; Harris *et al.*, 1996; Yamaoka *et al.* 1996]. The initial inflammatory response being mediated by the various bacterial toxins [Craig *et al.*, 1992; Neilsen & Andersen, 1992] as previously described. Soluble factors from activated monocytes are known to stimulate gastrin release *in vitro* [Golodner *et al.*, 1992; Lehmann *et al.*, 1996] and IL-1 β and TNF- α have been shown to induce gastrin release from primary cultures of rat [Weigert *et al.*, 1992], rabbit [Weigert *et al.*, 1996] and canine [Golodner *et al.*, 1993; Lehmann *et al.*, 1995] antral G cells. Therefore it is possible that activated macrophages stimulate the antral G cells to release gastrin *in vivo*. Evidence supporting this theory comes from results which show that reduction in gastrin release after eradication of *Helicobacter pylori* [Marshall *et al.*, 1988; Levi *et al.*, 1989b; Graham *et al.*, 1990] correlates with a decrease in mononuclear cells within the gastric mucosa rather than the clearance of neutrophils and bacterial

products [Graham *et al.*, 1993]. Whichever mechanism is responsible for hypergastrinaemia, however, cytokine levels in the antral mucosa are directly related to *Helicobacter pylori* infection and eradication of the bacterium, in patients with duodenal ulcer, leads to a marked drop in levels of IL-8 and TNF- α [Moss *et al.*, 1994].

7.0 RESULTS - DISCUSSION *IN VIVO* PROTEIN MARKERS OF SMALL BOWEL ULCERATION

7.1 RESULTS - *IN VIVO* PROTEIN MARKERS OF SMALL BOWEL ULCERATION

7.1.1 Results - Haptoglobin

7.1.1.1 Development of the Immunoelectrophoresis Procedure

The first gel using the DAKO rabbit anti-human haptoglobin was run using the antiserum at a concentration of 0.25 μ l/sq cm gel area, as recommended in the information leaflet supplied by DAKO, and this antiserum did cross react with the rat protein. The result was that the DAKO Human Serum Calibrator (DHSC) and the trial human serum produced nice clear rockets, with well defined peaks, which remained within the confines of the gel whereas the rat sera rockets were very faint and had run off the edge of the gel without forming a peak. However, it was possible to see that the control rat serum (pooled serum from four untreated rats from the Animal House, 0.25 ml per animal,) contained less haptoglobin than the serum from the rat with small bowel ulcers.

The experiment was repeated but this time the concentration of antibody was doubled to 0.50 μ l/sq cm gel area. This time the rockets formed by the DHSC and the trial human serum were shorter, darker and more compact as would be expected. The rockets formed by the rat sera were also easier to see and the control rat serum peaks had remained within the confines of the gel and at a 1/50 dilution were measurable. However, the track formed by the serum from the ulcerated rat still trailed off the gel at a 1/50 dilution and at 1/100 was not distinct enough to measure.

The experiment was repeated again but this time the gel contained 1% PEG 6000 as well as an antibody concentration of 50 μ l/sq cm gel area. (The immune complexes would precipitate out more rapidly in the presence of the PEG 6000.) This time the rockets formed by the DHSC were very short which confirmed that this particular serum would not be suitable for a standard curve. The peaks for the rat sera were bolder, shorter and more defined. The rockets formed by rat sera at a dilution of 1/100 were considered the clearest and easiest to measure for both the control rat serum and the ulcerated rat serum. On this particular gel an experimental standard curve, 1/12.5 to 1/100, had been

Well Number	Sample Number	Sera	ID Value	Dilution	Ulcers
36	Internal Control Serum	Sigma		1/100	
35	JR337	Normal Control		1/100	
34	JR335	Normal Control		1/100	
33	JR318	Normal Control		1/100	
32	JR303	Normal Control		1/100	
31	JR261	Indomethacin	65	1/100	*
30	JR260	Indomethacin	65	1/100	*
29	Standard 5	DAKO		1/160	
28	Standard 4	DAKO		1/80	
27	Standard 3	DAKO		1/40	
26	Standard 2	DAKO		1/20	
25	Standard 1	DAKO		1/10	
24	JR259	Indomethacin	65	1/100	*
23	JR258	Indomethacin	60	1/100	*
22	JR257	Indomethacin	60	1/100	*
21	JR256	Indomethacin	60	1/100	*
20	JR255	Indomethacin	65	1/100	*
19	JR254	Indomethacin	65	1/100	*
18	JR253	Indomethacin	65	1/100	*
17	JR230	Diclofenac	112.5	1/100	*
16	JR229	Diclofenac	112.5	1/100	*
15	JR228	Diclofenac	112.5	1/100	*
14	JR224	Diclofenac	100	1/100	*
13	JR222	Diclofenac	100	1/100	*
12	JR199	Vehicle Control		1/100	
11	Standard 1	DAKO		1/10	
10	Standard 2	DAKO		1/20	
9	Standard 3	DAKO		1/40	
8	Standard 4	DAKO		1/80	
7	Standard 5	DAKO		1/160	
6	JR195	Vehicle Control		1/100	
5	JR192	Vehicle Control		1/100	
4	JR191	Vehicle Control		1/100	
3	JR178	Normal Control		1/100	
2	JR176	Normal Control		1/100	
1	Internal Control Serum	Sigma		1/100	

Table 7.1.1.1 Description of Sera Run on Gel Reproduced on Facing Page

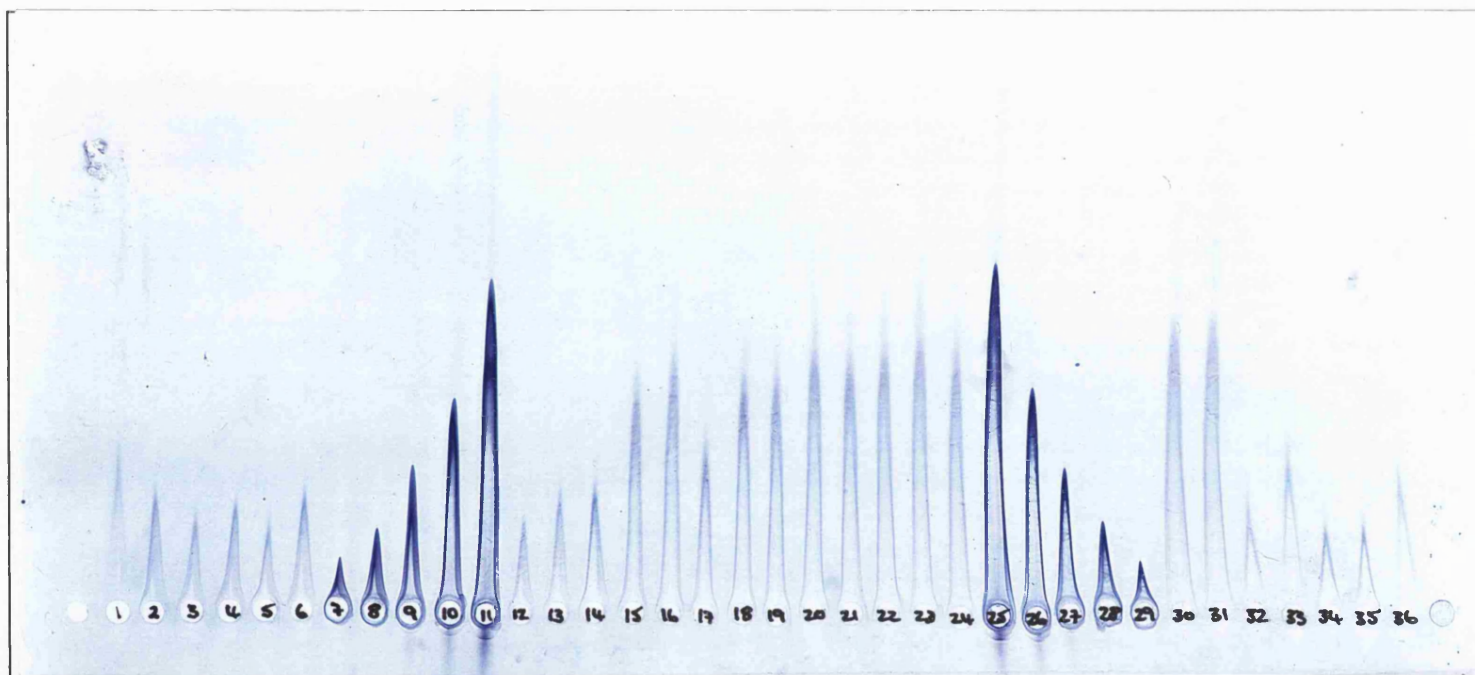


Photo 7.1.1 Example of Gel Produced During Rocket Immunoelectrophoresis Assay of Serum Haptoglobin (See Table 7.1.1.1)

run using pooled rat serum {Sigma} but this rat serum contained less haptoglobin than the control rats from the Animal House at an equivalent dilution. The experiment was repeated using the pooled rat serum {Sigma} at 100% and 1/6.25 for the highest points on the standard curve, however, on this occasion the tracks for the undiluted serum and the 1/6.25 dilution ran off the end of the gel.

It was decided to use the pooled rat serum from Sigma as a standard, 1/12.5 to 1/100, and to dilute the rat sera samples accordingly so that the peaks of the rockets fell within the confines of the standard curve. The samples were routinely diluted 1/50 and 1/100 but the serum from rats with severe ulceration had to be assayed at a 1/200 dilution. However, it was found that the value on the standard curve for a 1/50 dilution was more than double that obtained for a dilution of 1/100 and that the discrepancy increased between the 1/200 dilution and the 1/50. Therefore it was decided that the pooled rat serum {Sigma} was unsuitable for a standard curve and that it should be used as an internal control instead. Consequently each time a gel was run the value obtained for a 1/100 dilution of this serum showed whether the gel had worked correctly or not. Eventually the problem of a suitable serum for use as a standard curve was solved by DAKO who sent a human serum calibrator which had very high levels of haptoglobin.

Using these experimental parameters it was possible to run all the rat serum samples at a 1/100 μ l dilution so that the values obtained for each sample could be compared directly. Care was also taken to ensure that samples belonging to the same set of experiments were run on the same gel so that these too could be compared directly thus ensuring that any differences between them could not be due to an experimental artefact (photo. 7.1.1).

The serum haptoglobin concentration corresponding to the peak height was determined via a standard curve (see fig. 7.1.1.1) and the relative concentration calculated for each sample, taking the dilution factor into account. The results although expressed as mg/ml are not strictly accurate as the anti-human haptoglobin would not have bound to the rat protein with the same intensity as to the human serum that comprised the standard curve. However, the results are comparable within the rat serum samples.

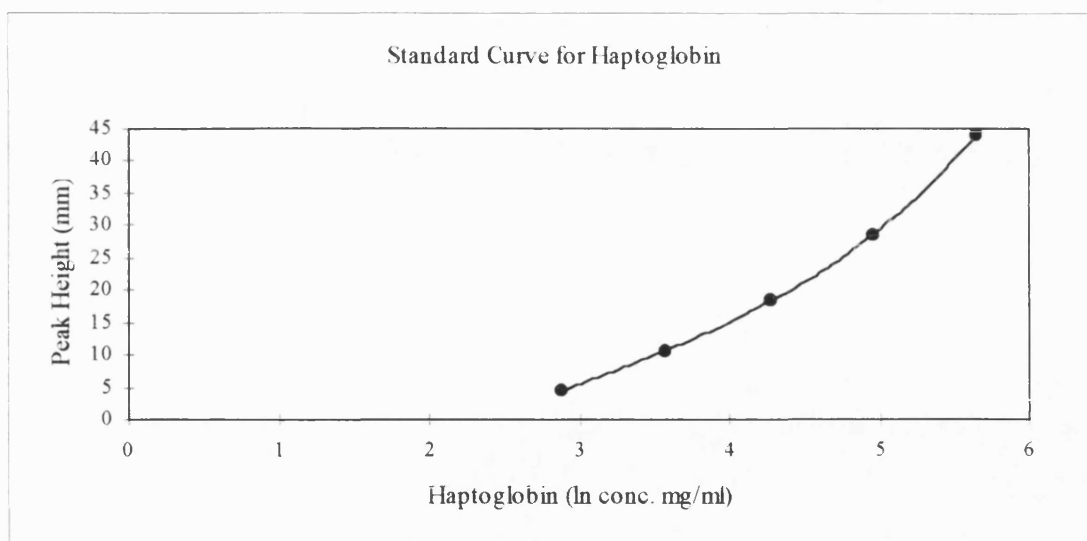


Figure 7.1.1.1 Standard Curve for Serum Haptoglobin

7.1.1.2 Correlation Between Ulceration and Serum Haptoglobin

There appeared to be a significant correlation ($r = 0.63$, $p < 0.001$) between the percentage area of total small bowel ulceration and the concentration of haptoglobin in the rat serum as measured by regression analysis (see fig. 7.1.1.2a).

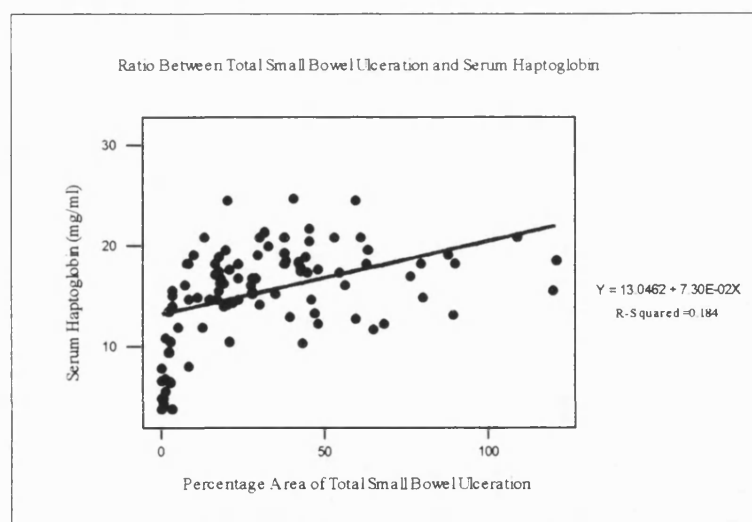


Figure 7.1.1.2a

However, it was noticed that animals treated with NSAIDs that did not develop ulcers had higher levels of serum haptoglobin than controls. It was decided to see if there was a correlation between the actual dose of an NSAID and the concentration of serum

haptoglobin. The only drug that this was possible with was indomethacin so the acute dose of indomethacin (mg/kg) was plotted against the concentration of serum haptoglobin. There appeared to be a better correlation ($r = 0.73$, $p < 0.001$) between these two parameters as measured by regression analysis (see fig. 7.1.1.2b).

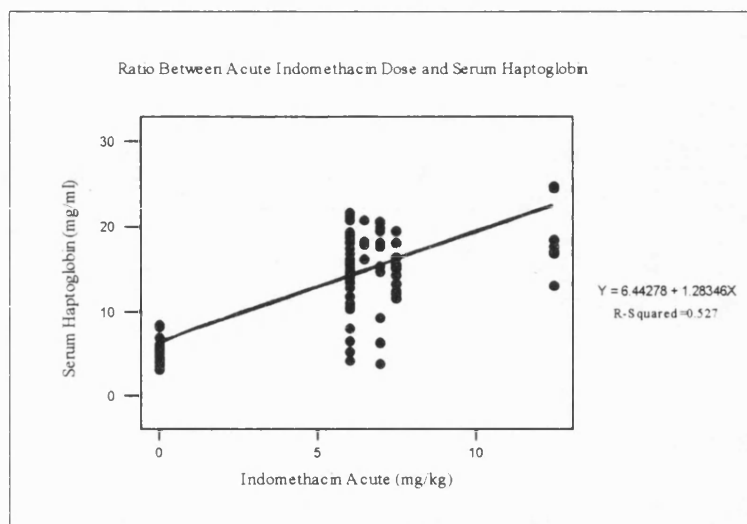


Figure 7.1.1.2b

7.1.1.3 Chronic Oral Administration of NSAIDs

As a result of the above correlation it was decided to analyse the serum haptoglobin concentrations found in the samples from animals without ulceration which had been dosed chronically with NSAIDs.

7.1.1.3.1 Short Term - Chronic Diclofenac

Animals had been dosed once daily for seven days either with diclofenac ID₇₅ (6.9 mg/kg) or diclofenac ID₁₀₀ (9.2 mg/kg) and killed on day ten. On comparing the concentration of haptoglobin, assayed in the serum of these animals, the concentration was found to be significantly higher ($p < 0.05$) in the animals dosed with diclofenac ID₁₀₀ (5.98 ± 0.25 mg/ml) than in the animals dosed with diclofenac ID₇₅ (4.54 ± 0.23 mg/ml). (Two of the animals dosed with diclofenac ID₁₀₀ did have one small ulcer each, equivalent to 0.15 per cent or 0.04 per cent of the total small bowel area respectively, but as the serum haptoglobin concentration was less in the animal with the larger area of

ulceration than the in the animal without any ulceration ($0.15\% \equiv 5.6 \text{ mg/ml}$: $0.0\% \equiv 5.75 \text{ mg/ml}$) it was deemed to be insignificant.) Animals which had been dosed chronically with diclofenac $ID_{112.5}$ (10.35 mg/kg) but for only four days had significantly higher ($p < 0.05$) levels of serum haptoglobin ($16.33 \pm 2.50 \text{ mg/ml}$) than the animals treated with the lower doses of diclofenac over a longer period of time. All these animals had ulcers in the small bowel but the mean total area of ulceration was only $1.31 \pm 0.21 \text{ mg/ml}$. The serum haptoglobin concentration found in the vehicle control animals dosed chronically with 10 per cent (v/v) Tween-80 (2.0 ml/kg) for seven days and killed on day ten was slightly higher ($4.84 \pm 0.90 \text{ mg/ml}$) than that found in control animals dosed concurrently with 2.0 ml/kg distilled water ($4.66 \pm 0.24 \text{ mg/ml}$). However this was not significant.

The serum haptoglobin concentration of $4.66 \pm 0.24 \text{ mg/ml}$ for control rats was high when compared with the report that concentration of haptoglobin in normal rats is 0.5 mg/ml [Wassdal *et al.*, 1991]. This is due to the fact that the anti-human haptoglobin did not bind so well to the rat protein which is why the peaks on the gel were fainter (see fig. 7.1.1.1a). These peaks would have been a lot shorter had the antiserum been more specific and this explains the elevated result. Although the values are approximately ten fold higher than what would be expected these results are still comparable between the animals used during the course of these experiments.

7.1.1.3.2 Long Term

The serum haptoglobin levels of the animals dosed with the ID_{125} values of fenbufen (50.0 mg/kg), nabumetone (79.0 mg/kg), naproxen (5.0 mg/kg) and piroxicam (7.0 mg/kg) for 21 days over a four week period were also investigated. The concentration of serum haptoglobin for the animals dosed with piroxicam ($10.08 \pm 1.65 \text{ mg/ml}$) was significantly higher ($p < 0.05$) than the values obtained for naproxen ($6.24 \pm 0.75 \text{ mg/ml}$), fenbufen ($5.04 \pm 0.1 \text{ mg/ml}$) and nabumetone ($4.83 \pm 0.37 \text{ mg/ml}$).

7.1.2 Results - Myoglobin

7.1.2.1 Isocratic System

Standards and serum samples were run on the isocratic system but during the course of the proceedings the pressure in the system began to decrease and the myoglobin peak was found to be getting broader, indicative of a 'hole' at the top of the column. The top was removed from the column and a gap of approximately 1.0 mm was found. The mesh was removed from the column and the column tested with the standard solution (supplied with the column) under test conditions. The subsequent chromatogram obtained was identical to that supplied with the column which showed that the column had not deteriorated during the 40 hours that it had been in use. The procedure was halted until the problem with the column could be resolved.

Although this system was only used for a short period of time it did provide some useful information. Analysis was conducted to determine whether or not serum myoglobin was stable in distilled water, as the serum samples would have to be diluted prior to injecting them onto the column. (It had been reported that myoglobin was very unstable and that it was at its most stable within 100 per cent serum [Powell *et al.*, 1984].) A comparison between myoglobin standards diluted either with serum or with distilled water revealed that myoglobin was stable in distilled water (see figs. 7.1.2.1a and 7.1.2.1b).

During the course of this work it was found that levels of myoglobin in rat serum were very low so it was decided to collect urine for analysis instead as it was reported that myoglobin levels in urine were generally higher [Powell *et al.* 1984]. In addition to this urine samples were 'cleaner' than serum samples which meant that higher concentrations of sample could be passed through the column without the risk of it becoming blocked. It was also decided that the separation may be improved if the analysis was conducted using a gradient system. Consequently myoglobin analysis was suspended until suitable apparatus was available, a gradient system and a replacement column, and sufficient urine samples had been collected.

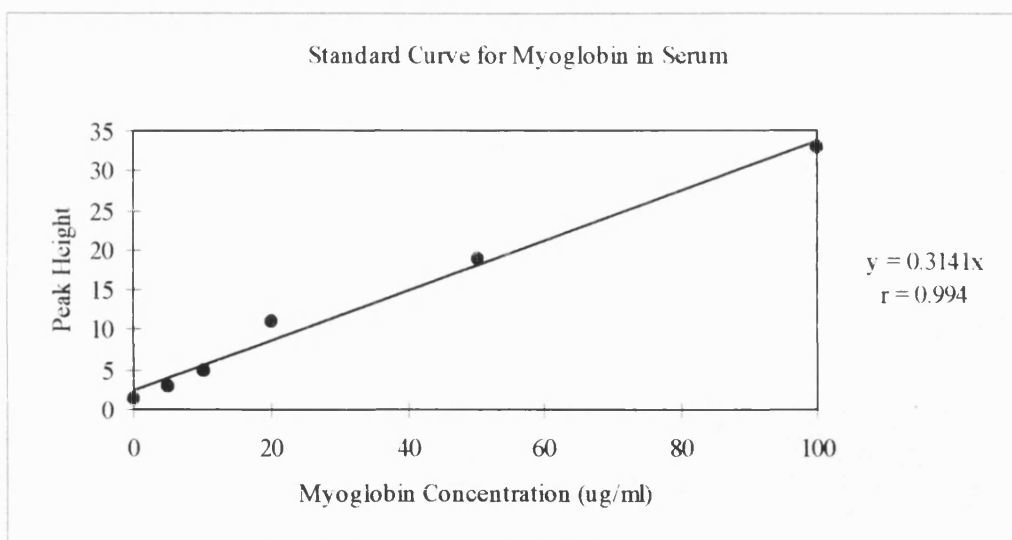


Figure 7.1.2.1a Standard Curve for Myoglobin in Serum

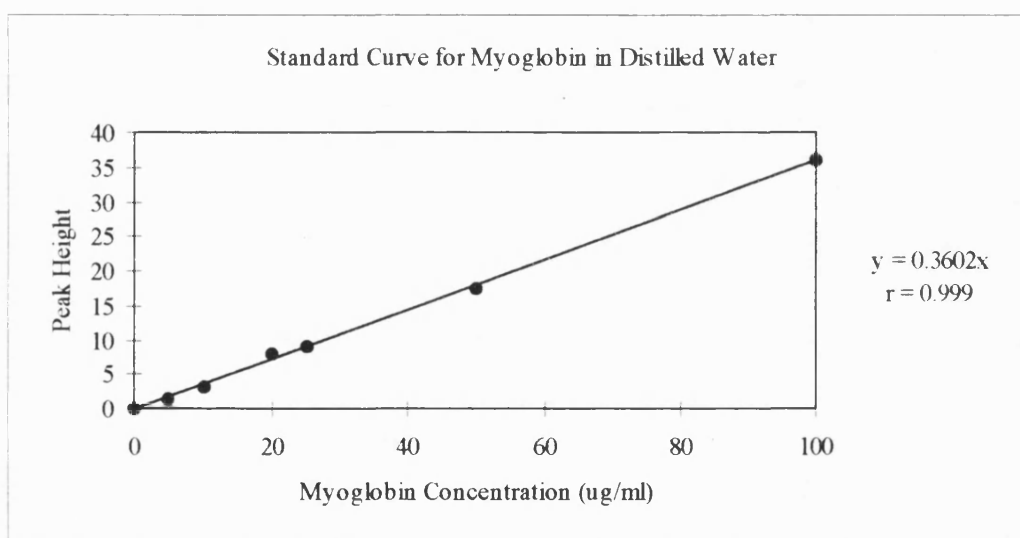


Figure 7.1.2.1b Standard Curve for Myoglobin in Distilled Water

Although only a few samples had been analysed ($n = 15$) there did appear to be a trend as samples from animals without ulcers had more serum myoglobin than animals with small bowel ulceration, however, the correlation was not significant. (Pooled control rat serum (0.25 ml/rat) from four animals provided the highest myoglobin result of 25.63 $\mu\text{g/ml}$.)

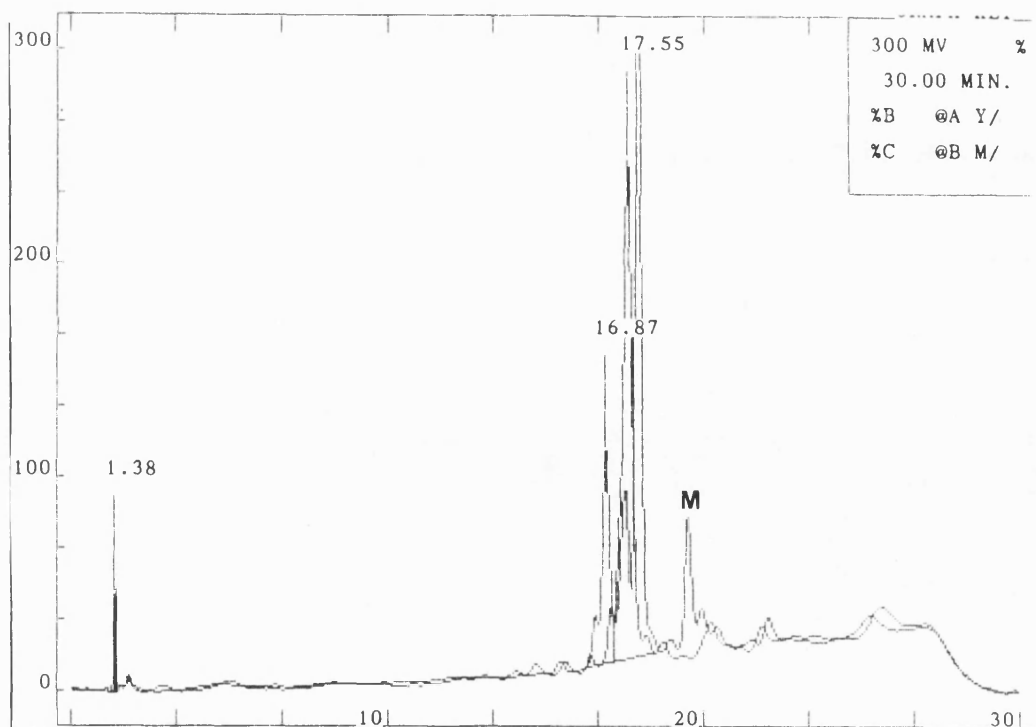


Figure 7.1.2.2.a

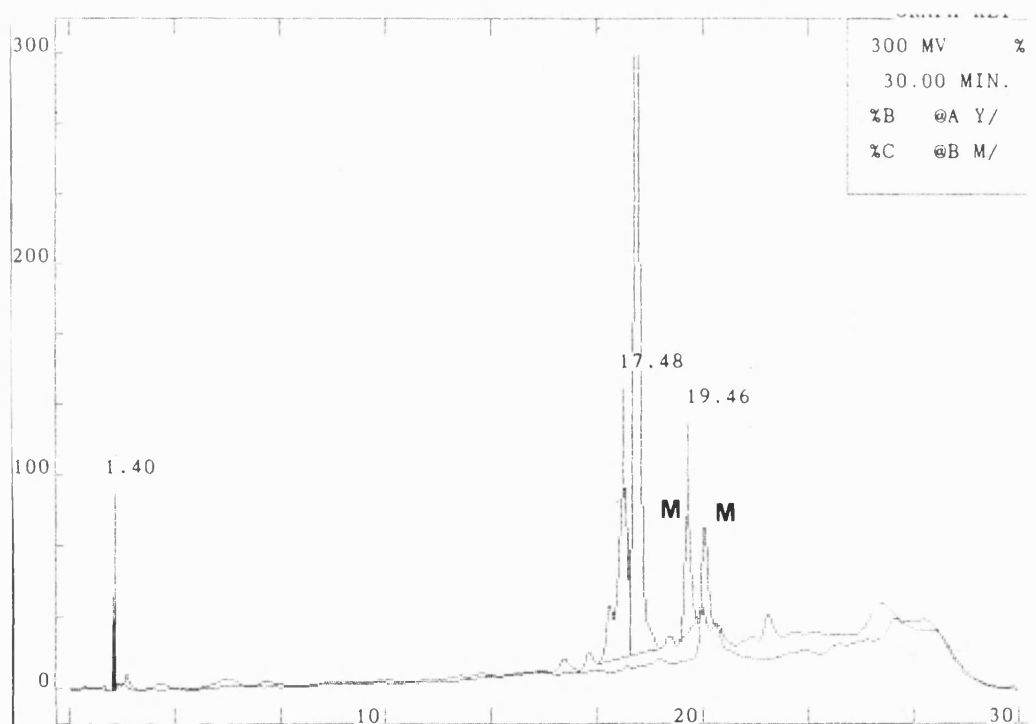


Figure 7.1.2.2.b

Figure 7.1.22 Chromatograms Illustrating Lack of Serum Myoglobin Within Rat Serum Samples

a. Sample Superimposed With Sample Spiked With Myoglobin (100 μ g/ml)

b. Sample Spiked With Myoglobin (100 μ g/ml) Superimposed With Myoglobin Standard (100 μ g/ml)

M = Myoglobin Peaks

7.1.2.2 Gradient System

Initially a few rat and human urine samples were run on this system to assess its suitability and then it was decided to postpone all further analysis until the end of the project so that all the samples could be run at the same time. Owing to the scarcity of rat urine samples, as a consequence of a lack of success in urine collection, serum samples had to be used. However, when using a 100 µl loop, with the serum sample diluted 1:9 with filtered distilled water, the myoglobin peak was undetectable (see fig. 7.1.2.2a and 7.1.2.2b). As a result neat serum was used but the protein tended to block the column. In order to try and facilitate analysis, the serum was filtered using a micropartition system {MPS-1, Amicon} but this filtered out all the protein which had been seen to be eluting from the column.

There was always a problem in that the system never seemed to stabilise as the peaks produced on the chromatogram by the myoglobin standards appeared progressively later as the protein took longer to elute each time a standard was injected. This may have been due to the fact that the column required a longer time than usual to become 'loaded' and as availability of the HPLC equipment was limited to short periods of time (two weeks) when it was not being used by other researchers the gradient system procedure probably never stabilised. Unfortunately at this point all work in the laboratory had to cease as it was decided that insufficient time was available for any more practical work. As a consequence no further analysis was conducted on these samples.

7.2 DISCUSSION - *IN VIVO* PROTEIN MARKERS OF SMALL BOWEL

ULCERATION

7.2.1 Discussion - Haptoglobin

Haptoglobin, an acute phase glycoprotein with a molecular weight of approximately 90 kDa, has a tetrameric $\beta\alpha\alpha\beta$ structure, where the α -chains are linked by a disulphide bond. These α -chains are each linked to a β -chain by an additional disulphide bond [Malchy *et al.*, 1983]. Acute phase proteins are a group of mainly glycoproteins which are synthesised by hepatocytes [Putnam, 1975] in response to cytokines produced by leucocytes and macrophages which have themselves been stimulated by infection or inflammation [Bornstein, 1982; Marinkovic *et al.*, 1989; Kent, 1992]. Thus serum haptoglobin concentrations increase after inflammatory stimuli [Murray & Connell, 1960; Bowman & Kurosky, 1982; Eckersall & Conner, 1988; Morimatsu *et al.*, 1992], which is why it is used as an indicator of inflammatory diseases [Bowman & Kurosky, 1982; Heinrich *et al.*, 1990].

Haptoglobin is also believed to have antiinflammatory properties as it has been shown to decrease both neutrophil metabolism [Oh *et al.*, 1990a; El Ghmati *et al.*, 1996], the chemotactic response of monocytes and to inhibit the proliferation of peripheral blood mononuclear cells [Samak *et al.*, 1982]. Heterologous haptoglobin may also modulate B cell proliferation and decrease antibody production [Oh *et al.*, 1990b]. Haptoglobin is also thought to be a natural bacteriostat [Eaton *et al.*, 1982] by preventing the utilisation of haemoglobin iron by various pathogenic bacteria, especially those which may be present in abscesses and those which escape into the peritoneal cavity from a perforated ulcer [Eaton *et al.*, 1982].

Haptoglobin as a Possible Indicator of Small Bowel Ulceration

There was a significant correlation ($r = 0.63 \equiv p < 0.001$) between the percentage area of total small bowel ulceration and the concentration of haptoglobin in the rat serum (see fig. 7.1.1.2a). However, the correlation between an actual acute dose of indomethacin and the concentration of serum haptoglobin was more significant ($r = 0.73$, $p < 0.001$). Similarly animals which had been dosed chronically with diclofenac for seven days

without developing ulceration had significantly ($p < 0.05$) higher concentrations of serum haptoglobin than control animals. The serum haptoglobin levels of the animals dosed chronically with the ID_{125} values of fenbufen, nabumetone and naproxen for 21 days over a four week period were not significantly different to those found in control animals ($p > 0.05$), although the value obtained for naproxen (6.24 ± 0.75 mg/ml) was slightly higher than those obtained for fenbufen (5.04 ± 0.1 mg/ml) and nabumetone (4.83 ± 0.37 mg/ml). However, the concentration of serum haptoglobin for the animals dosed over a four week period with piroxicam (10.08 ± 1.65 mg/ml) was significantly higher ($p < 0.05$). Therefore there would appear to be a greater correlation between the serum haptoglobin level and an inflammatory response within the animal rather than the degree of ulceration.

Evidence which would appear to support this supposition comes from the experiments investigating the role of cytokines in ulcer formation. The lowest levels of the inflammatory cytokines, TNF- α and IL-1 α , were found within the intestinal mucosa of the animals which had developed small bowel ulcers. The animals given indomethacin ID_{30} , however, a dose which did not produce ulcers, had the highest levels of IL-1 α within the small bowel tissue accompanied by many mononuclear cells within the lamina propria, all indicative of an inflammatory response.

These results correlate well with the ulcerogenic properties of the drugs under investigation. Indomethacin was found to be the most toxic of the NSAIDs investigated during this project followed by diclofenac and the serum haptoglobin levels reflect this. The degree of gastrointestinal damage caused by an NSAID appears to be related to the degree of enterohepatic recirculation that the drug undergoes and the pKa value. Thus the most damage is caused by indomethacin and diclofenac which have a combination of significant enterohepatic recirculation in conjunction with a low pKa value. The haptoglobin results obtained from the long term dosing experiment, however, would appear to indicate that it is the extent to which a drug undergoes enterohepatic recirculation which is critical to ulcer formation within the small bowel.

Animals dosed with indomethacin ID₃₀ would appear to illustrate that an inflammatory response within the small bowel, as a result of NSAID ingestion, is indicative of imminent ulcerogenesis. Thus the serum haptoglobin levels found in animals dosed long term with piroxicam would appear to indicate that there was a distinct possibility that the animals would have developed small bowel ulcers if treatment had continued. These results would appear to show that after indomethacin and diclofenac, piroxicam is the most toxic NSAID of the drugs investigated. Thus in spite of piroxicam having the highest pKa value of the drugs used during this project it is the fact that it undergoes greater enterohepatic recirculation than the remaining drugs (naproxen, fenbufen and nabumetone) that results in the increased inflammation. The serum haptoglobin concentration for animals dosed long term with naproxen was slightly higher than that found in control animals and this is probably due to its low pKa value. The fact that naproxen undergoes significantly less enterohepatic recirculation within the rat than indomethacin, diclofenac and piroxicam, is what makes it less toxic.

The serum haptoglobin level of fenbufen treated animals (5.04 ± 0.1 mg/ml) was slightly higher than that of nabumetone (4.83 ± 0.37 mg/ml) although this was not significant ($p > 0.05$). However, during this particular experiment (long term chronic dosing) the animals dosed with fenbufen gained the least weight over the four week period. The mean weight gain between the groups was not significantly different although the animals given fenbufen ID₁₂₅ weighed significantly less ($p < 0.05$) than the animals dosed with naproxen ID₁₂₅ (140.00 ± 20.08 g versus 185.00 ± 8.10 g). This result may be indicative of the fact that although fenbufen is quite innocuous when compared to indomethacin, diclofenac, piroxicam and naproxen, it is more toxic than nabumetone. Therefore, comparing the six NSAIDs used during this research nabumetone causes the least side effects followed by fenbufen, naproxen, piroxicam, diclofenac and indomethacin.

Levels of serum haptoglobin would appear to be a useful *in vivo* protein marker of small bowel inflammation which could be an indicator of those at risk of developing a small bowel ulcer when taking NSAIDs long term. The serum haptoglobin levels would probably still be elevated once an ulcer had formed, although from the experiments

within the rat model it would appear that serum haptoglobin levels begin to fall in the presence of small bowel ulceration.

Patients suffering with rheumatoid arthritis have increased serum haptoglobin concentrations when compared to normal individuals [Hallgren *et al.*, 1985; Kirstein & Mathieson, 1987] and this is due to the tissue inflammation which is associated with the disease. The serum haptoglobin results obtained in the rat model after NSAID administration would seem to suggest that increased levels in patients with rheumatoid arthritis may also be due in part to long term ingestion of NSAIDs.

7.2.2 Discussion - Myoglobin

Myoglobin, a relatively small oxygen-binding protein with a molecular weight of about 16.7 kDa, is found primarily in muscle cells [Lehninger, 1982]. Both myoglobin and haemoglobin can be liberated at sites of tissue injury [Odeh, 1991; Halliwell, *et al.*, 1992] such as that seen in small bowel ulceration or in the inflamed rheumatoid joint [Blake *et al.*, 1981]. Antiinflammatory drugs [Evans *et al.*, 1994], particularly indomethacin have been shown to be capable of generating damaging radicals [Vaananen *et al.*, 1991] and this process is possibly mediated via myoglobin which is thought to act as a catalyst for the formation of strong oxidants [Evans *et al.*, 1994; Phung *et al.*, 1994].

The lack of results for this part of the project was very disappointing. There did appear to be a trend although this correlation ($r = 0.36$) was not significant ($r < 0.48 \equiv p > 0.05$) within the number of samples ($n = 15$) analysed. The animals with small bowel ulcers tended to have serum myoglobin levels which were lower than those found in control animals. Similarly animals given an NSAID which had not developed small bowel ulceration had levels lower than the control animals although the serum myoglobin concentration was more than that found in the animals with small bowel ulcers. Further investigation of the change in serum myoglobin concentration as a result of ulceration is probably worthwhile.

8.0 MECHANISMS INVOLVED IN ULCERATION OF THE STOMACH AND SMALL BOWEL : GENERAL DISCUSSION

Synthetic NSAIDs have been available for almost 100 years the first, acetylsalicylic acid (aspirin), being manufactured in 1899 [Wallace, 1997]. Although being very efficacious in the treatment of arthritis and other musculoskeletal disorders, long term use of acetylsalicylic acid was found to be acutely toxic to the gastric and duodenal mucosa [Levi & Shaw-Smith, 1994] and consequently pharmaceutical companies across the world began conducting research into finding a more innocuous alternative. As a result there are now currently 23 NSAIDs available for use in the UK alone [Levi & Shaw-Smith, 1994] and the world market for NSAIDs now exceeds £4 billion per year [Garner, 1992; Wallace, 1997]. Although the newer NSAIDs are less damaging to the stomach it would appear that many of them have harmful side-effects throughout the remainder of the gastrointestinal tract [Sturges & Krone, 1973; Schwartz, 1981; Hawkey, 1990].

The process of NSAID-induced ulceration would appear to be extremely complex with the entire procedure being dependent on many separate contributory mechanisms, the removal of just one of these parameters being sufficient to prevent ulcerogenesis. The first mechanism of damage to be determined was the ability of NSAIDs to inhibit the enzyme cyclooxygenase (COX-1 and COX-2), the first dedicated enzyme in prostaglandin (PG) synthesis. Unfortunately it is also through the inhibition of this enzyme that NSAIDs mediate their antiinflammatory properties [Vane, 1971]. Prostaglandins of the E and F series have been shown to be instrumental in initiating some of the local and systemic characteristics of inflammation, such as vasodilation, increased vascular permeability, swelling, increased leucocyte migration and pain [Swingle & Kvam, 1986]. Prostaglandins are also believed to be one of the mediators of rheumatoid arthritis so it would be expected that drugs which inhibit their synthesis would ameliorate arthritic diseases [Lewis & Keft, 1995]. The problem is that prostaglandins and their properties are numerous, they affect almost every biological system and many of the functions they exhibit are diametrically opposed to one another [Robert, 1979]. Prostaglandins are necessary for the maintenance of the epithelial cells within the gastrointestinal tract [Miller & Jacobson, 1979; Robert, 1979] where they

protect the gastrointestinal epithelium against a variety of noxious agents and thus they confer a cytoprotective effect [Miller & Jacobson, 1979; Robert, 1979]. Therefore when rats are dosed with prednisolone, an inhibitor of the enzyme phospholipase A, which prevents the release of arachidonic acid from the cell membrane [Gryglewski *et al.*, 1975] they develop multiple necrotic ulcers in the ileum [Lancaster & Robert, 1978], similar to those caused by indomethacin. In this instance it is the lack of prostaglandins which is responsible for the small bowel damage and this is indicative of their importance in maintaining the integrity of the gastrointestinal epithelium. Consequently a similar result would be expected when their production is prevented by NSAID inhibition of cyclooxygenase (COX-1 and COX-2).

The inhibition of cyclooxygenase (COX-1 and COX-2) by NSAIDs results in similar damage occurring, although in this instance the damage is more severe. Inhibition of phospholipase A by prednisolone results in a lack of arachidonic acid which means that neither prostaglandins nor leukotrienes can be formed. However, when cyclooxygenase (COX-1 and COX-2) is inhibited by NSAIDs, the production of leukotrienes is increased as the surplus arachidonic acid is channelled into their production via the enzyme 5-lipoxygenase [Rainsford, 1987, 1993a; Bjarnason *et al.*, 1989]. This overproduction of leukotrienes, in conjunction with a depletion in cellular prostaglandins, results in an inflammatory response as leukotrienes are chemoattractants for a number of mononuclear cells [Kuby, 1997b]. This inflammatory process can be attenuated either by the concomitant administration of PGE₂ or one of its analogues [Robert, 1974, 1975, 1979; Fang *et al.*, 1977; Tabata & Okabe, 1983; Romain *et al.*, 1987] or by an inhibitor of 5-lipoxygenase [Rainsford, 1987; Rainsford, 1993b].

This particular mechanism is a property of the older NSAIDs such as indomethacin, diclofenac and naproxen which tend to inhibit COX-1, the constitutive form of cyclooxygenase, rather than COX-2, the inducible form. COX-1 is the enzyme responsible for maintaining the integrity of cells within various organs and other physiological tissues [Funk *et al.*, 1991; Simmons *et al.*, 1991]. Thus inhibition of COX-1 leads to the small bowel damage which is associated with NSAIDs. The newer NSAIDs, such as nabumetone, selectively inhibit COX-2 [Meade *et al.*, 1993] which is

the inducible form of the enzyme that plays a key role in the inflammatory response [Vane *et al.*, 1994]. COX-2 is found predominantly in leucocytes, such as macrophages and monocytes, in which *de novo* synthesis of the COX-2 gene occurs in response to IL-1 [Hla & Neulson, 1992]. NSAID-induced small bowel damage can therefore be prevented by administering a newer, COX-2 inhibitor, such as nabumetone, rather than an older style NSAID which is an inhibitor of COX-1.

The initial damage caused by some NSAIDs is probably due to a direct topical effect of the drug as it enters the stomach and then, if not fully absorbed there, as it passes through into the small bowel. This topical damage within the stomach occurs as a result of 'ion trapping' and is caused primarily with NSAIDs which have a low pKa value, such as diclofenac, naproxen and indomethacin. These drugs, which are predominantly un-ionised within the acidic environment of the gastric lumen, pass through the lipid bilayer of the cells which constitute the gastric epithelium. Once they enter the cytosol of a gastric epithelial cell, where the pH is maintained at about 7.4, they become ionised with the result that they become trapped within the cell. NSAIDs which have a higher pKa value, such as piroxicam and fenbufen, or are neutral like nabumetone do not become trapped within the gastric epithelial cells as they remain predominantly un-ionised even within the cytosol of the epithelial cells. As a result they are free to pass back and forth across the lipid bilayer without causing very much damage to the cell. To a lesser extent the process of 'ion trapping' continues within the small bowel even though most of the drugs with the lower pKa values are ionised at pH 7.0. A proportion of these drugs, however, will still be un-ionised and it is this portion that is able to pass through into the cells lining the intestinal epithelium where they become trapped. The drugs with the higher pKa values are still un-ionised and are thus able to pass freely back and forth across the lipid bilayer and consequently they cause little damage.

Once inside the cells of the gastrointestinal epithelium the NSAIDs may cause damage through more than one mechanism. In the stomach, where the water soluble NSAIDs, such as diclofenac, can become highly concentrated, albeit temporarily, within the gastric epithelial cells, the high intracellular concentration of acidic NSAID may exert a direct acid damaging effect [Schoen & Vender, 1989]. NSAIDs are also able to uncouple

mitochondrial oxidative phosphorylation and it has been reported that the NSAIDs with the lower pKa values are the most effective uncouplers [Mahmud *et al.*, 1996a]. Topical damage as a result of this process is more significant within the intestinal epithelium where the pH of the small bowel prevents high concentrations of drug accumulating through 'ion trapping'. In the presence of such uncouplers electron transport proceeds normally but ATP is not formed by the mitochondrial ATPase [Stryer, 1981]. This depletion in ATP is believed to be responsible for the small bowel permeability, a characteristic of NSAID-induced damage, as ATP is necessary for the maintenance of the tight junctions within the intestinal epithelium [Meza *et al.*, 1980; Madara & Dharmasathaphorn, 1985; Madara *et al.*, 1986, 1987]. Additional damage within the cell could be due to NSAID inhibition of various enzymes involved in glycolysis and/or the tricarboxylic acid cycle which also prevents the formation of ATP [Smith & Dawkins, 1971].

This topical damage would appear to be exacerbated the more times the gastrointestinal epithelium is exposed to the drug. In the case of a single dose, the gastric epithelium can only be exposed to the drug once prior to absorption, but this is not the case with the intestinal epithelium. Therefore the enterohepatic circulation plays an important role in NSAID-induced ulceration within the small bowel and here it is only the drugs which are excreted in the bile that have ulcerogenic properties. Thus as a result of enterohepatic recirculation the small bowel is exposed to the effects of a single dose on more than one occasion with some NSAIDs. The NSAIDs with the lower molecular weights, such as naproxen, fenbufen and nabumetone, which do not undergo significant enterohepatic recirculation, only pass through the small bowel once. Thus any damage that the drug may cause via a direct topical effect can only occur once prior to absorption. The NSAIDs with the higher molecular weights, such as diclofenac, indomethacin and piroxicam, all undergo enterohepatic recirculation and as a result the small bowel is exposed to the effects of a single dose on more than one occasion.

Although the phenomenon of 'ion-trapping' occurs primarily within in the gastric lumen, a proportion of the drugs with the lower pKa values will still be un-ionised. The un-ionised portion is therefore able to pass through the lipid bilayers of the intestinal

epithelial cells where it becomes trapped within the cytosol of the cell. The damage caused as a result of 'ion-trapping' is relatively insignificant if the intestinal epithelium is only exposed to the drug once but if exposed continually this damage can become significant. This is probably why naproxen, which has a lower pKa value than indomethacin and is readily trapped within gastrointestinal epithelial cells, is less ulcerogenic than diclofenac and indomethacin. NSAIDs that undergo enterohepatic recirculation, such as piroxicam, which have a higher pKa value are less likely to be trapped within the cells of the intestinal epithelium. Thus even if the small bowel is exposed to drugs such as these on numerous occasions the resultant topical damage will be very slight. This is probably why piroxicam is less ulcerogenic than diclofenac and indomethacin. During this project indomethacin and diclofenac were the only drugs which caused small bowel ulceration in the rat and this was probably due to a combination of their having low pKa values in addition to undergoing extensive enterohepatic circulation. Evidence supporting this proposal is provided by the fact that a chemical modification of diclofenac, which does not undergo such extensive enterohepatic recirculation, is considerably less ulcerogenic [Reuter *et al.*, 1997]. Diclofenac is less toxic to the intestinal epithelium in man than indomethacin, even though it has a lower pKa value, and in humans this can be explained by the fact that indomethacin undergoes more extensive enterohepatic recirculation in man than diclofenac. This does not explain the difference in ulcerogenesis observed within the rat where both drugs undergo enterohepatic recirculation to a similar extent [Hucker *et al.*, 1966; Yesair *et al.*, 1970; Duggan *et al.*, 1975; Riess *et al.*, 1975]. In this instance the difference is probably due to the fact that diclofenac sequesters arachidonic acid into lipid (largely triglyceride) pools [Day *et al.*, 1990] from where it can be re-incorporated into the inner membrane of the cell, thus limiting leukotriene formation.

In rats the role of normal gastrointestinal bacteria is probably the major contributory factor in NSAID-induced ulceration. In the experimental rat small bowel tissue which had been taken from animals treated with indomethacin ID₆₀, the discrete ulcers and erosions were associated with Peyer's patches. Peyer's patches are areas of attraction for many bacteria [Garland *et al.*, 1979; Savage, 1979; Sonnenwirth, 1980; Wolf, 1988; O'Loughlin, 1995; Sansonetti *et al.*, 1996; Kuby, 1997] as they are able to adhere to the

smooth surface of the M cells which cover the lymphoid follicles [Savage, 1969; Klaasen *et al.*, 1992; Jepson *et al.*, 1993; Sansonetti *et al.*, 1996]. However, the majority of the intestinal epithelium within the small bowel, of both man and the rat, is devoid of bacteria as gut motility, a non-immunological defence mechanism [Sarker & Gyr, 1992], prevents bacteria from adhering to the epithelial cells which are covered in microvilli.

In the experimental ulcerated rat small bowel tissue, the areas of damage away from Peyer's patches had not elicited an inflammatory response. Thus it would appear that, in the absence of bacteria and their toxins, such damage would be repaired via epithelial cell turnover and would not result in an ulcer. Similarly, indomethacin-induced small bowel permeability on its own does not automatically result in an ulcer. It is probably the bacteria and/or their toxins entering the extracellular space of the intestinal epithelium which initiates the catastrophic inflammatory cytokine mediated response. Evidence supporting this proposal is provided by the fact that germ-free rats do not develop ulcers when given an ulcerogenic dose of indomethacin which would produce ulcers in conventional rats [Robert & Asano, 1977]. The total removal of these microorganisms from the gastrointestinal tract with a combination of the antibiotics metronidazole and amoxycillin, prior to dosing with indomethacin, conferred complete protection against the ulcerogenic process mediated by the NSAID. The antibiotic treatment was still effective to some degree even when the animals were dosed up to two weeks before the indomethacin challenge. This protection has also been observed by other researchers in animals pretreated with antibiotics or given them concomitantly [Kent *et al.*, 1969; Fang *et al.*, 1977; Yamada *et al.*, 1993; Collins *et al.*, 1995]. Antibiotics given after the ulcerative process has already been initiated, however, confers no significant protection at all and does not promote healing [Kent *et al.*, 1969; Robert & Asano, 1977; Satoh *et al.*, 1983; Benoni *et al.*, 1987; Bjarnason *et al.*, 1988b]. It would seem from this that once the process of ulcerogenesis has started, mechanisms other than those afforded by bacteria play a key role.

Neutrophils have been implicated as being the key contributors to the tissue damage seen in the small bowel as a result of NSAID-induced ulceration [Del Soldato *et al.*, 1985; Pihan *et al.*, 1987; Vaananan *et al.*, 1991]. Animals which are neutropaenic or have been

made neutropaenic by treatment with an anti-neutrophil antiserum are resistant to NSAID-induced ulceration [Wallace *et al.*, 1990; Lee *et al.*, 1992]. The recruitment of these leucocytes to the area of infection is most probably triggered by bacterial toxins, such as lipopolysaccharide, diffusing into the gastrointestinal mucosa as a result of the NSAID-induced small bowel permeability where they initiate the inflammatory cytokine cascade. Once this inflammatory response has been initiated, however, it would appear to become self generating, especially in the presence of NSAIDs which are inhibitors of COX-1 and as a result of this property are responsible for the increased production of chemoattractant leukotrienes. The necrotic tissue within the area of ulceration contains a mixture of potent chemoattractant debris made up of bacterial products and fragments of cells [Kuby, 1997a] which have been destroyed as a result of neutrophil degranulation [Grisham & Granger, 1988]. Thus the eradication of bacteria from the area of ulceration, once this process has started, would not appear to be able to prevent the inflammatory response. This is probably why administration of antibiotics after the ulcerative process has started does not heal the ulcers but there is a possibility that antibiotics may attenuate the process to a certain extent. (It is possible that antibiotics given to rats 12-24 hours after an acute dose of indomethacin ID₁₂₅ (12.5 mg/kg) may have prevented the longitudinal ulcers forming.)

Humans are less likely than rats to be exposed to large amounts of bacteria within the small bowel as man does not practice coprophagy, however, bacteria here probably do have a similar role in ulcerogenesis. It has been reported that long-term use of NSAIDs can result in diaphragm-like small bowel strictures [Bjarnason *et al.*, 1988a; Levi *et al.*, 1990], usually within the ileum [Lang *et al.*, 1988; Levi *et al.*, 1990], which may narrow the lumen down to a pinhole [Bjarnason *et al.*, 1993; Levi & Shaw-Smith, 1994] and these strictures are often associated with circumferential ulceration [Lang *et al.*, 1988]. It may be that bacteria, which would otherwise pass through the small bowel harmlessly due to normal gut motility, become trapped in the area of these strictures enabling them to colonise a region usually sparsely populated by bacteria. (Under normal conditions the upper/mid regions of the small bowel are usually free from *Bacteroides spp.* but these bacteria have been found in large numbers within areas of stasis in the ileum [Gorbach *et al.*, 1969; Rath *et al.*, 1996].) Thus bacteria within these small bowel strictures in

conjunction with small bowel permeability, due to NSAID ingestion, may be responsible for the formation of these circumferential ulcers.

In recent years it has become widely known that it is a bacterium which is responsible for 80 per cent of gastric ulcers and 95 per cent of ulcers in the duodenum [Peterson, 1991; Graham, 1991; Veldhuyzen van Zanten *et al.*, 1994]. The ulcerative process in the case of *Helicobacter pylori* is probably also dependent on mechanisms other than those exhibited by the bacterium itself, as illustrated by the fact that very few of the people infected with the more virulent strains go on to develop gastric or duodenal ulcers [Sipponen *et al.*, 1989]. Factors contributing to ulcer formation in this instance are many and people who are *Helicobacter pylori*-positive are more at risk from developing an ulcer if they are taking NSAIDs [Jones *et al.*, 1991] as a result of mechanisms previously discussed. *Helicobacter pylori*-positive subjects are also at risk of developing an ulcer and/or gastric cancer if they smoke, consume alcohol or have a stressful lifestyle and the mechanisms involved in these risk factors are described in the Introduction.

Thus it would appear that the development of an ulcer within the gastrointestinal tract does depend on a balance between protective and aggressive factors, as previously proposed [Shay, 1961] and when this balance is disturbed by outside deleterious influences, such as NSAIDs or *Helicobacter pylori*, ulceration occurs. The normal complement of gastrointestinal microflora would in themselves appear to be 'potential pathogens' which are kept in check by defence mechanisms which have evolved within the gut to protect the host from serious infections [Kagnoff, 1987b]. In the past it has been proposed that the normal microflora within the gastrointestinal tract is essential to life [Schottelius, 1902] and many scientists still believe this to be true. However, this hypothesis has long since been disproved by generations of germ-free animals outliving their conventional laboratory counterparts [Reyniers *et al.*, 1949; Gordon & Pesti, 1971; Mackowiak, 1982].

This project has investigated various mechanisms by which lesions of the stomach and small bowel are produced in rat and man. Differences in ulcerogenic properties between the selected NSAIDs have been established and comparisons made between the two

species. In the rat indomethacin and diclofenac are extremely ulcerogenic as enterohepatic recirculation of these drugs is considerable within this species, however, in man the enterohepatic recirculation is less extensive. Consequently NSAID-induced ulceration does not occur as readily in man where ulcer formation in the stomach and small bowel may be mediated by exogenous agents or environmental factors such as smoking, ethanol and stress. *Helicobacter pylori* is specific for the stomach and the duodenum whereas NSAIDs cause damage throughout the gastrointestinal tract. Once diagnosed, ulcers associated with *Helicobacter pylori* are treated with antibiotics whereas NSAID-induced ulcers are treated either by withdrawal of the drug for six months or by substitution with another.

In comparison with man the rat displays many physiological differences, such as drug metabolism and inflammatory response, and behavioural differences such as coprophagy which results in there being a significant difference in the composition of small bowel microflora between the two species. Interestingly it has proved virtually impossible to infect the stomach of rats with *Helicobacter pylori*. In conclusion it would appear that these differences preclude the rat from being a suitable model for NSAID-induced ulceration in man.

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Appendix 1

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SEM PROCESSING

SM1

Name				Date			
Julia Reid				15/9/93			
Tissue Type	(Also Human Duodenum)						
CIA = Rat Small Bowel - Acetone treated to remove mucus							
C1 = Rat Small Bowel - Warm saline treated to remove mucus							
C3 = Rat Small Bowel - Lesion							
Prefix				Time	Over Night		
2% GDA in 0.05M Sörensens Buffer pH 7.4				Temp	4°C		
Wash				Time	20 mins		
0.1M Sörensens Buffer +				Temp	RT		
0.15M Sucrose pH 7.4				Temp	RT		
Postfix				Time	1 hour		
1% OsO ₄ in 0.1M Sörensens Buffer +				Temp	RT		
0.15M Sucrose pH 7.4				Temp	RT		
Wash				Time	15 mins		
Distilled water					(x 6 changes)		
(Follow with OTOTO Method)				Temp	RT		
Distilled water (Over Night)							
Dehydrate				Time	10 mins each		
					(x 3 changes)		
50, 60, 70, 85, 90, 95, 100, 100% dry				Temp	RT		
Infiltrate - CO ₂ CPD				Time			
				Time			
Acetone:CO ₂				Temp			
				Time			
Drying							
Critical-Point Drying Method (Appendix 2)							

CRITICAL-POINT DRYING METHOD

1. Ensure that the pressure chamber temperature is between about 5 and 12°C by circulating cold water through the water jacket or setting and switching on the thermoelectric cooling/heating device.
2. Pressure-test the VitonTM seals around the windows and door of the pressure chamber by opening the chamber to the cylinder, thereby ensuring that the chamber is at the same pressure as the cylinder (approx 800 psi/5.5 MPa). This should be done prior to committing valuable specimens to the chamber and finding later that a seal is leaking and that the run must be aborted.
3. Load the dehydrated specimens, soaked in intermediate fluid (100%) acetone), into the specimen-holders of the critical-point dryer.
4. Insert the specimen-holder into the pressure chamber and seal the door.
5. Fill the chamber with liquid carbon dioxide. The intermediate fluid should sit as a reasonably discrete and separate layer in the bottom of the pressure chamber.
6. Withdraw intermediate fluid from the bottom of the pressure chamber via the liquid bleed valve until the level of the transitional fluid (the gas/liquid interface) is just above the specimen-holder.
7. Refill the chamber with liquid carbon dioxide, and repeat step 5 about four times over a period of about 10 min in order to flush out all residual intermediate fluid (acetone).
8. Leave the specimens soaking in carbon dioxide for 20-45 min, depending upon their hardness and size, so that the last traces of acetone may diffuse from them. Gentle rocking of the critical-point dryer will cause turbulence in the liquid

carbon dioxide and increase the diffusion rate, but this agitation should not be too violent; otherwise mechanical damage to the specimens could occur.

9. Seal the pressure chamber and gently open the liquid bleed valve until the chamber is only three-quarters full of liquid carbon dioxide.
10. Close the liquid bleed valve.
11. At this stage the chamber will be at the same pressure as the carbon dioxide cylinder (approx 800 psi/5.5 MPa). gently warm the pressure chamber by either slowly increasing the temperature of the water in the jacket surrounding the chamber or by altering the settings of the thermoelectric cooling/heating device. The temperature should be raised to 38°C over a period of about 20 min. The pressure will also increase and should be maintained at about 1300-1400 psi(9.0-9.7 MPa) by gently opening and closing the gas bleed valve.
12. Once the critical point has been exceeded (38°C and 1300 psi/9.0 MPa), gently open the gas bleed valve and depressurise the bomb over a period of about 10-15 min. As far as possible, the gas should flow from the chamber at a constant rate.
13. Remove the specimen-holders and transfer them to a desiccator.

Ref: Lewis, E.R., Jackson, L. & Scott, T. (1975) Comparison of miscibilities and critical-point drying properties of various intermediate and transitional fluids. In *Scanning Electron Microscopy*. Pt II. eds. Johari, O & Corvin, I. pp. 392-317. Chicago: ITT Research Inst.

Source: Robards, A.W., Wilson, A.J. *et al.*, (1993) Basic biological preparation techniques for scanning electron microscopy. (Section 11). In *Procedures In Electron Microscopy*. eds. Robards, A.W. & Wilson, A.J. pp. 11:4.3-11:4.4. Chichester: John Wiley & Sons Ltd.

Appendix 3.1

Centre for Electron Optical Studies
School of Materials Science
Claverton Down
Bath
BA2 7AY

TEM PROCESSING

Name		Date	
Julia Reid - Method 6		18/7/95	
Tissue Type			
Rat Small Bowel (Control and Experimental)			
Prefix	Time		5 hrs
2% GDA in 0.05M Sörensens Phosphate Buffer + 0.02M Sucrose pH 7.2 385 mOsm		Temp	On Ice
Wash	Time		15 mins (x 3 Changes)
0.01M Sörensens Phosphate Buffer 0.16M Sucrose pH 7.2 385 mOsm		Temp	On Ice
Postfix	Time		1.0 hr
1% OsO ₄ in 0.1M Sörensens Phosphate Buffer + 0.16M Sucrose pH 7.2 385 mOsm		Temp	On Ice
Wash	Time		Overnight
0.1M Sörensens Phosphate Buffer + 0.16M Sucrose pH 7.2 385 mOsm		Temp	4°C
Dehydrate	Time		15 mins each (x 2 changes)
In Acetone 60, 80, 90, 95, 100, 100% dry		Temp	60-90% on ice 90-100% RT
Infiltrate	1:1	Time	1.5 hrs
In Spurr's Resin : Acetone	3:1	Time	1.0 hr
	100%	Time	1.5 hrs
	100%	Time	Overnight
	100%	Time	1.0 hr
Embed	In Spurr's Resin		
Polymerise	At 70°C for 8 Hour		

Appendix 3.2a₁

Centre for Electron Optical Studies
School of Materials Science
Claverton Down
Bath
BA2 7AY

TEM PROCESSING

Name		Date	
Julia Reid - Method 7a ₁		23/10/96	
Tissue Type			
Rat Small Bowel (Control)			
Prefix	(by perfusion)	Time	2½ hrs
2% GDA in 0.05M Sörensens Phosphate Buffer		Temp	RT
0.02M Sucrose			
pH 7.2 371mOsm			
Wash		Time	15 mins
0.1M Sörensens + 0.16 M Sucrose			(x 3 changes)
pH 7.2 384/385 mOsm		Temp	RT
Postfix		Time	1 hour
1% OsO ₄ in 0.1 M Sörensens + 0.16 M Sucrose		Temp	RT
pH 7.2 384/385 mOsm			
Wash		Time	15 mins each
0.1M Sörensens + 0.16M Sucrose			(x 3 changes)
pH 7.2 384/385 mOsm		Temp	RT
Dehydrate		Time	15 mins each
In Acetone			(x 3 changes)
60,80,90,95,100%,		Temp	RT
100% dry x 2			
Infiltrate	in Spurr's	1:1	Time 1 hr x 4 changes
Resin:Acetone		3:1	Time 1 hr x 4 changes
		100%	Time O.N.
			Time
Embed	in Spurr's Resin		
Polymerise	8 - 9 hrs 70°C		

Appendix 3.2a₂

Centre for Electron Optical Studies
School of Materials Science
Claverton Down
Bath
BA2 7AY

TEM PROCESSING

Name		Date	
Julia Reid - Method 7a ₂		23/10/96	
Tissue Type			
Rat Small Bowel (Control)			
Prefix	(by perfusion)	Time	2½ hrs
2% GDA in 0.05M Sörensens Phosphate Buffer		Temp	RT
0.02M Sucrose			
pH 7.2 371mOsm			
Wash		Time	15 mins
0.1M Sörensens + 0.16 M Sucrose			(x 3 changes)
pH 7.2 384/385 mOsm		Temp	RT
Postfix		Time	1 hour
1% OsO ₄ in 0.1 M Sörensens + 0.16 M Sucrose		Temp	RT
pH 7.2 384/385 mOsm			
Wash		Time	15 mins each
0.1M Sörensens + 0.16M Sucrose			(x 3 changes)
pH 7.2 384/385 mOsm		Temp	1 @ RT+2 @ RT
			O.N. 4°C
Dehydrate		Time	15 mins each
In Acetone			(x 3 changes)
60,80,90,95,100%		Temp	RT
100% dry x 2			
Infiltrate	in Spurr's	1:1	Time 1 hr x 1 change
Resin:Acetone		3:1	Time 1 hr x 1 change
		100 %	Time 1 hr
		Time	
Embed	in Spurr's Resin		
Polymerise	8 - 9 hrs 70°C		

Appendix 3.2b

Centre for Electron Optical Studies
School of Materials Science
Claverton Down
Bath
BA2 7AY

TEM PROCESSING

Name		Date	
Julia Reid - Method 7b		22/10/96	
Tissue Type			
Rat Small Bowel (Control)			
Prefix	(by perfusion)	Time	2½ hrs
2% GDA in 0.05M Sörensens Phosphate Buffer		Temp	RT
0.02M Sucrose			
pH 7.2 371mOsm			
Wash		Time	15 mins
0.1M Sörensens + 0.16 M Sucrose			(x 3 changes)
pH 7.2 384/385 mOsm		Temp	RT
Postfix		Time	1 hour
1% OsO ₄ in 0.1 M Sörensens + 0.16 M Sucrose		Temp	RT
pH 7.2 384/385 mOsm			
Wash		Time	15 mins each
0.1M Sörensens + 0.16M Sucrose			(x 3 changes)
pH 7.2 384/385 mOsm		Temp	R.T
Dehydrate	In Quetol	Time	15 mins each
60,70,85,90,95,100%			(x 2 changes)
		Temp	RT
Infiltrate		3:1	Time 1.0 hr
Quetol:Full Resin Mix		100%	Time 5.0 hr
			Time
			Time
Embed	Quetol/NSA/MNA Resin		
Polymerise	at 60°C for 24 hrs		

Appendix 4

STAINING PROCEDURES FOR TRANSMISSION ELECTRON MICROSCOPY

Appendix 4a

URANYL ACETATE

Uranyl Acetate Solution

0.6 g Uranyl Acetate

20 ml 30% Ethanol (in sterile distilled water)

In a fume cupboard, carefully add the 30% ethanol to the uranyl acetate and mix. Filter the solution and then store in an amber glass bottle in the dark. This solution will keep for up to three weeks

Uranyl Acetate Staining Procedure

See below.

LEAD CITRATE

Appendix 4b

Reynold's Lead Citrate Solution

1.33 g Lead Acetate

1.76 g Sodium Citrate

30 ml Sterile Distilled Water

In a fume cupboard, place the lead acetate and the sodium acetate in a 50 ml volumetric flask and add the sterile distilled water. Shake vigorously for one minute (until the compounds are fully into solution) then leave to stand for 30 minutes (shaking once every five to ten minutes). Add 8.0 ml 1.0M sodium hydroxide solution (carbonate free) then make up to 50 ml with sterile distilled water. This solution will keep for up to four weeks.

Reynold's Lead Citrate Staining Procedure

See below.

Uranyl Acetate / Reynold's Lead Citrate Staining Procedure

In a fume cupboard, aliquot drops of boiled glass distilled water, uranyl acetate solution and lead citrate solution onto different pieces of dental wax. Place the grids, section side down, onto the droplets of boiled glass distilled water (one grid per droplet). Dry grids carefully with a piece of filter paper and then place on the droplets of uranyl acetate (one grid per droplet) and leave for 10 minutes. Dry grids carefully with a piece of filter paper and then place on the droplets of lead citrate solution (one grid per droplet). Leave for one to five minutes. Place the grids, section side down, onto fresh droplets of boiled glass distilled water (one grid per droplet). Rinse grids well with running sterile distilled water, dry with filter paper and store in a grid box.

N.B. Uranyl acetate must be kept away from light as much as possible so keep the droplets which are on the dental wax covered at all times (wrap aluminium foil around a small glass trough). The lead citrate must be maintained in an atmosphere free of carbon dioxide so keep the dental wax that the drops of lead citrate solution are on in a covered glass petri dish with filter paper soaked in 10.0M sodium hydroxide.

Appendix 4c

Double Lead Stain Solution

0.25 g Lead Citrate

0.1-0.2 g One Sodium Hydroxide Pellet

In a fume cupboard, pour 40 ml of sterile distilled water into a 50 ml volumetric flask and then add the sodium hydroxide pellet and shake until dissolved. Add lead citrate and make up to 50 ml with sterile distilled water, shake until dissolved. This solution, which has a pH of about 12, will keep for several months in a sealed container at 4°C.

N.B. Filter or centrifuge before use.

Uranyl Acetate / Double Lead Stain Staining Procedure

In a fume cupboard, aliquot drops of boiled glass distilled water, uranyl acetate solution and lead citrate solution onto different pieces of dental wax. Place the grids, section side down, onto the droplets of boiled glass distilled water (one grid per droplet). Dry grids carefully with a piece of filter paper and then place, section side down, on the droplets of lead citrate solution (one grid per droplet) leave for one to five minutes. Rinse each grid several times in a beaker of sterile distilled water. Dry grids carefully with a piece of filter paper and then place, section side down, on the droplets of uranyl acetate (one grid per droplet) and leave for 40 minutes. Rinse each grid several times in a beaker of sterile distilled water then dry carefully with filter paper. Place grids, section side down, on fresh droplets of lead citrate solution (one grid per droplet) and leave for 20 minutes. Rinse each grid several times in a beaker of sterile distilled water, dry with filter paper and store in a grid box.

N.B. Uranyl acetate must be kept away from light as much as possible so keep the droplets which are on the dental wax covered at all times (wrap aluminium foil around a small glass trough). The lead citrate must be maintained in an atmosphere free of carbon dioxide so keep the dental wax that the drops of lead citrate solution are on in a covered glass petri dish with filter paper soaked in 10.0M sodium hydroxide.

Appendix 5

HAEMATOXYLIN AND EOSIN STAINING

The method described here is typical of the many H & E procedures.

1. De-wax and hydrate paraffin sections. Frozen sections should be dried onto slides.
2. 3% Acetic Acid in Absolute Alcohol (60 sec).
3. 95% Alcohol (60 sec).
4. Tap Water (60 sec).
5. Mayer's Haematoxylin (60 sec).
6. Water (60 sec).
7. Alkaline Water (60 sec).
8. Tap Water (60 sec).
9. Eosin (60 sec).
10. Tap Water (60 sec).
11. Absolute Alcohol (60 sec).
12. Absolute Alcohol (60 sec).
13. CitrocLEAR (60 sec).
14. Mount in XAM.

Results

Nuclear chromatin stains blue to purple and cytoplasm, collagen, keratin, erythrocytes stain pink

HAEMATOXYLIN AND EOSIN STAINING SOLUTIONS

1. MAYERS HAEMATOXYLIN

1.0g Haematoxylin Monohydrate

0.2g Sodium Iodate

50.0g Aluminium Potassium Sulphate 12-Hydrate (Potassium Alum)

1.0g Citric Acid

50.0g Chloral Hydrate

1000ml Distilled Water

In a fume cupboard, using a 2000ml glass beaker, dissolve the haematoxylin, aluminium potassium sulphate 12-hydrate and sodium iodate in the distilled water by leaving overnight or by stirring. Once these reagents have dissolved (warm if necessary) add the chloral hydrate and the citric acid then boil for 5 min. Once cooled, filter then store at room temperature.

This solution will keep for up to a year but some batches lose their potency after a few months. The solution may be used many times but if it fails to stain nuclei properly, it may be over-oxidised. Should this occur make up the solution as before but halve the concentration of sodium iodate.

2. EOSIN

5.0g Eosin

Make up to 1000ml with distilled water.

This solution keeps indefinitely and may be used repeatedly. Moulds often grow in this solution and on such occasions the microorganisms need to be removed by filtration.

Ref. Kiernan, J.A. (1981). Histological Staining in One or Two Colours. In *Histological & Histochemical Methods: Theory and Practice*. pp. 77-87. Oxford: Pergamon Press.

AVIDIN-BIOTIN METHOD FOR PARAFFIN SECTIONS

USING MOUSE OR HAMSTER ANTISERA (MONOCLONALS)

1. Pick up sections onto glass slides, precoated with 'Sta-On', dry overnight at 37°C.

Before commencing dewaxing steps, put 300 ml of distilled water and 300 ml Tris Buffered HCl (for trypsinisation step) into the oven to warm to 37°C.

2. Dewax sections 30 minutes take to alcohol.

- a. CitrocLEAR 1 for 10 mins.
- b. CitrocLEAR 2 for 10 mins.
- c. CitrocLEAR 3 for 10 mins.
- d. Alcohol (industrial methylated spirits O.K.) for 10 mins.

(Usually only need one 10 min 'dunk' in CitrocLEAR. Use three here to be particularly careful. Probably could make do with two. At the end of the week throw away the citrocLEAR in the first pot. Move other two pots forward, so that final pot of citrocLEAR is always fresh.)

After deparaffinisation can mark round section with a wax pen. However have to be careful not to do it too close to the section as the antibody will shrink away from the wax mark. If you have a lot of sections use this method for speed. However if only a few best just to wipe carefully around the section with a clean dry tissue after adding solutions or washing. (Wipe under the slide first then carefully around section.) Both procedures prevent the antibody from spreading out all over the slide.

During these washes prepare blocking solution for Step 3. Also add chymotrypsin and calcium chloride to tris buffered HCl which is at 37°C. Also need to begin preparing primary antibody dilutions i.e. work out concentrations required and label tubes. Do not add antibody to dilution buffer (TBS) too soon before use.

3. Block in 2.5% hydrogen peroxide in methanol for 10 min.

Put 300 mls of highgrade methanol into a pot. To this add 7.5 ml of 30% H₂O₂ (100 vols), 2.5 ml at a time using a 3.0 ml plastic pasteur pipette. Stir solution with the pipette.

(This is the 'blocking reagent' which blocks the peroxidases which are usually present. This has to be done because we use the enzyme horse radish peroxidase to label the antigen/antibody complex. It is possible to use the enzyme alkaline phosphatase instead, but cannot store these sections long term as you can with ones that have been prepared using horseradish peroxidase.)

4. Wash in running tap water for 2 min followed by a quick dunk in a pot of distilled water.

5.a Place slides which are to be trypsinised into distilled water which is at 37°C and leave in the oven for 5 min.

5.b Place slides which are not to be trypsinised into glass pot filled with tris buffered saline (TBS).

6. Place slides with sections which need to be trypsinised in chymotrypsin solution, preheated to 37°C, for 15 min in the oven.

(When tissue is placed in formalin, bonds binding the tissue proteins together are formed. This is the preserving process as it prevents autolysis by immobilising all tissue proteins such as enzymes etc. The trypsin cleaves these bonds so that those antigens which are locked inside can be exposed to the antibody. However some antigens do not survive trypsin so these sections by-pass this step.

7. Take sections which have been in chymotrypsin solution out of the oven and wash in running water for 2 min followed by a quick dunk in a pot of distilled water. Then place sections in pot of TBS.

Trypsin can be used again if doing another run on the same day. However needs to be made up fresh each day.

8. Wipe carefully around each section then place in moist chamber, section covered with TBS. (Sections must not be allowed to dry out.)

9. Treat with 5% Normal Rabbit Serum for 5 min.

(Normal tissue contains other connective tissues such as collagen. These have an affinity for the antibody, non-specific binding. As a result these sites have to be blocked. To do this use whole serum from the appropriate animal i.e. use the serum from the species that

the secondary antibody has been raised in. This blocks out interference which can occur by the secondary antibody binding to sites in the tissue and swamping the antigen/antibody effect. After this treatment the secondary antibody should only bind to the primary antibody.)

10. Tap off. DO NOT WASH.

Tip off excess serum

11. Incubate in Primary Antisera for 1 hour.

Allow 100 µl per small section. With larger sections may need to increase volume containing amount of antibody required accordingly. Use a 1.0 ml plastic pasteur pipette to aliquot antibody (a couple of drops \equiv 100 µl). Negative controls just get 100 µl TBS.

N.B. Put the sections into the slide tray/box before you add the primary antibody. Put on the lid and leave in a warm place (or in the incubator at 37°C depending on assay). Must ensure that the sections do not dry out. If using DAKO tray make sure the reservoir has TBS in it. If using a box (Shandon) put a tissue soaked in TBS along the bottom of the box at the top of the glass slides (white border at the bottom so can read what is written on the slides).

12. Gently rinse in TBS from wash bottle.

13. Incubate in secondary antibody for 40 min.

For monoclonals the secondary antibody is either Rabbit Anti-Mouse Biotinylated (Blue) or Rabbit Anti-Hamster Biotinylated (Yellow). This has to be diluted 1/400 in TBS. Make up sufficient quantity so that there is 100µl per section. Use a 1.0ml plastic pasteur pipette to aliquot diluted antibody (a couple of drops \equiv 100µl). Leave sections in a warm place or in the incubator at 37°C.

14. Prepare Avidin Biotin Complex (ABC).

ABC must be made up at least 30 min before use as it has to complex. Make up in the ratio 10:10:1000 A:B:TBS and store at 4°C until required. 1000 µl is sufficient for 5 sections.

15. Gently rinse twice in TBS from wash bottle.

16. Add ABC and leave at room temperature for 40 min.

Aliquot 200 µl ABC per section with plastic pasteur pipette (about 4 drops).

Make up DAB during this incubation time. (DAB is very nasty so take care and make up and use solution in a fumehood.)

17. Gently rinse twice in TBS from a wash bottle.

18. Aliquot 200-300 µl of DAB onto each section and leave for 10 min.

19. Remove DAB with a disposable pasteur pipette into waste bottle. Rinse sections with 200-300 µl distilled water and remove this with pasteur pipette into waste bottle.

20. Place sections in a rack which is standing in a pot of tap water, then wash in running tap water for 5 min.

21. Stain nuclei in Gills haematoxylin for 1.0 min.

Can re-use Gills solution many times. Only need to change once staining begins to get faint. However if there is a shiny film on the surface remove this before you immerse the slide rack by using a folded tissue to skim the surface. If the Gills has been used a lot or left over the weekend filter it (filter paper and funnel is sufficient).

22. Blue in tap water. (Do this by placing rack in a pot of running tap water). In Bath, because the local water supply is alkaline, all you have to do is wash in running tap water for about 10 mins. This causes the haematoxylin stain to turn blue. If in an area where the water is more acidic, need to use a solution of bicarbonate.

N.B. Empty first lot of water out immediately as otherwise the sections can effectively sit in haematoxylin longer if the concentration is greater at the bottom.

23. Dehydrate sections.

Dry off rack with a tissue. Dunk in industrial alcohol twice (a few seconds in each pot). Then put rack into another pot of citroclear. (Don't have to leave the sections in here too long. Just ensure that the citroclear on the sections runs clear before they are mounted.

24. Mount sections in XAM.

Place cover slips of suitable sizes onto tissue. Put a blob of XAM on the cover slip, using a 3.0 ml plastic pasteur pipette, one blob/section. Put slide, still 'runny' with citrocLEAR (ie take slide out of rack which is still in the citrocLEAR, stand slide up on end on pile of green paper towels just to drain off excess citrocLEAR) on top of the coverslip. Turn slide over and allow weight of cover slip to spread out the XAM and displace the air bubbles. Any persistent air bubbles can be forced out from under the coverslip with a slight amount of pressure. Once all the coverslips are on can dry off with a cloth or tissue.

N.B. Make sure that you put the cover slips on the correct side of the slide.

IMMUNO PEROXIDASE SOLUTIONS

1. BLOCKING REAGENT (2.5% HYDROGEN PEROXIDE IN METHANOL)

7.5ml 30% Hydrogen Peroxide (100 vol) [H_2O_2] FW:- 34.01

300ml Methanol (HPLC) [CH_3OH] FW:- 32.04

Using a 3.0ml plastic pasteur pipette add 3×2.5 ml hydrogen peroxide to the methanol and mix by stirring with the pipette.

30% Hydrogen Peroxide BDH:- 101284N

Methanol Fisons:- M/4056/17

2. HYDROCHLORIC ACID 2.0M

172.0ml Hydrochloric Acid (S.G. 1.18) [HCl] FW:-36.46

Add concentrated hydrochloric acid to 750.0ml distilled water and make up to 1.0 litre with distilled water. Store at 27°C .

Hydrochloric Acid (S.G. 1.18) Fisons:- H/1150/PB17

3. TRIS BUFFERED SALINE (TBS):0.05M Tris/Hcl, 0.15M NaCl, pH 7.6

30.285g Tris(hydroxymethyl)methylamine [$\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$] FW:- 121.14

42.5g Sodium Chloride [NaCl] FW:- 58.44

115.5ml 2.0M Hydrochloric Acid [HCl]

Make up to 5.0 litres with distilled water. pH to 7.6 with Sodium Hydroxide. Store at 27°C .

Tris(hydroxymethyl)methylamine BDH:- 103156X

Sodium Chloride Fisons:- S/3120/63

4. TRIS BUFFERED HCl :0.05M, pH 7.8 (for trypsin)

15.0g Tris(hydroxymethyl)methylamine [$\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$] FW:- 121.14

47.0ml 2.0M Hydrochloric Acid [HCl]

Make up to 2.5 litres with distilled water. pH to 7.8 with Sodium Hydroxide.

Tris(hydroxymethyl)methylamine

BDH:- 103156X

5. TRYPSIN SOLUTION

300ml Tris Buffered HCL pH 7.8

0.3g α -Chymotrypsin

0.3g Calcium Chloride $[\text{CaCl}_2 \cdot 2\text{H}_2\text{O}]$

FW:- 147.02

Warm 300 ml of tris buffered HCL to 37°C in an oven. Take glass pot containing warm tris buffered saline and place on a magnetic stirrer (heater on very low) with a flea. Weigh out calcium chloride and add to tris buffered HCL then weigh out trypsin (best to do this in a fume cupboard as some people can become sensitised to it very rapidly causing breathing problems!) and add to the tris buffered HCL, remove reagent adhering to the weighing boat by immersing it in the solution. Once reagents have dissolved adjust the pH to 7.8 with 3% sodium hydroxide Return to oven at 37°C.

α -Chymotrypsin (Type II: From Bovine Pancreas)

Sigma:- C-4129

Calcium Chloride

BDH:- 10070

6. NORMAL RABBIT SERUM 5% (Monoclonals)

200ml Tris Buffered Saline pH7.6

0.2g Sodium Azide

10.0ml Normal Rabbit Serum

Weigh out Sodium Azide in a fume cupboard (POISON - WEAR GLOVES) and add to Tris Buffered Saline, mix. Add Normal Rabbit Serum to this solution and mix. Store at 4°C.

Sodium Azide

BDH:- 10369

Normal Rabbit Serum

Dako:- X0902

7. NORMAL SWINE SERUM 5% (POLYCLONALS)

200ml Tris Buffered Saline pH 7.6

0.2g Sodium Azide [

10.0ml Normal Swine Serum

Weigh out Sodium Azide in a fume cupboard (POISON - WEAR GLOVES) and add to Tris Buffered Saline, mix. Add Normal Swine Serum to this solution and mix. Store at 4°C.

Sodium Azide

BDH:- 10369

Normal Swine Serum

Dako:- X0901

8.AVIDIN-BIOTIN COMPLEX

1000µl Tris Buffered Saline pH 7.6

10µl Reagent A

10µl Reagent B

Mix and leave in the fridge for 40 min before use. (1000µl is sufficient for 5 slides.)

ABComplex

Dako:- K355

9. DAB REAGENT

15.0ml Tris Buffered Saline pH 7.6

10mg 3,3'-Diaminobenzidine Tetrahydrochloride [$C_{12}H_{14}N_4 \cdot 4HCl$] (1 tablet)

12.0µl 30% Hydrogen Peroxide (100 vol) [H_2O_2] FW:- 34.01

In a fume cupboard add a DAB tablet to a universal containing Tris Buffered Saline, vortex mix until the DAB has dissolved. Do not add Hydrogen Peroxide until just before use.

3,3'-Diaminobenzidine Tetrahydrochloride

Sigma:- D-5905

30% Hydrogen Peroxide

BDH:- 101284N

AVIDIN-BIOTIN METHOD FOR PARAFFIN SECTIONS

USING RABBIT ANTISERA (POLYCLONALS)

1. Pick up sections onto glass slides, precoated with 'Sta-On', dry at 37°C overnight.

Before commencing dewaxing steps, put 300ml of Tris Buffered HCl (for trypsinisation step) into the oven to warm to 37°C.

2. Dewax sections 30 minutes take to alcohol.
 - a. CitrocLEAR 1 for 10 mins.
 - b. CitrocLEAR 2 for 10 mins.
 - c. CitrocLEAR 3 for 10 mins.
 - d. Alcohol (industrial methylated spirits O.K.) for 10 mins.

(Usually only need one 10 min 'dunk' in CitrocLEAR. Use three here to be particularly careful. Probably could make do with two. At the end of the week throw away the citrocLEAR in the first pot. Move other two pots forward, so that final pot of citrocLEAR is always fresh.)

After deparaffinisation can mark round section with a wax pen. However have to be careful not to do it too close to the section as the antibody will shrink away from the wax mark. If you have a lot of sections use this method for speed. However if only a few best just to wipe carefully around the section with a clean dry tissue after adding solutions or washing. (Wipe under the slide first then carefully around section.) Both procedures prevent the antibody from spreading out all over the slide.

During these washes prepare blocking solution for Step 3. Also add chymotrypsin and calcium chloride to tris buffered HCl which is at 37°C. Also need to begin preparing primary antibody dilutions i.e. work out concentrations required and label tubes. Do not add antibody to dilution buffer (TBS) too soon before use.

3. Block in 2.5% hydrogen peroxide in methanol for 10 min.

Put 300 mls of highgrade methanol into a pot. To this add 7.5 ml of 30% H₂O₂ (100 vols), 2.5 ml at a time using a 3.0 ml plastic pasteur pipette. Stir solution with the pipette.

(This is the 'blocking reagent' which blocks the peroxidases which are usually present. This has to be done because we use the enzyme horse radish peroxidase to label the antigen/antibody complex. It is possible to use the enzyme alkaline phosphatase instead, but cannot store these sections long term as you can with ones that have been prepared using horseradish peroxidase.)

4. Wash in running tap water for 2 min followed by a quick dunk in a pot of distilled water.

5.a Place slides which are to be trypsinised into distilled water which is at 37°C and leave in the oven for 5 min.

5.b Place slides which are not to be trypsinised into glass pot filled with tris buffered saline (TBS).

6. Place slides with sections which need to be trypsinised in chymotrypsin solution, preheated to 37°C, for 15 min in the oven.

(When tissue is placed in formalin, bonds binding the tissue proteins together are formed. This is the preserving process as it prevents autolysis by immobilising all tissue proteins such as enzymes etc. The trypsin cleaves these bonds so that those antigens which are locked inside can be exposed to the antibody. However some antigens do not survive trypsin so these sections by-pass this step.

7. Take sections which have been in chymotrypsin solution out of the oven and wash in running water for 2 min followed by a quick dunk in a pot of distilled water. Then place sections in pot of TBS.

Trypsin can be used again if doing another run on the same day. However needs to be made up fresh each day.

8. Wipe carefully around each section then place in moist chamber, section covered with TBS. (Sections must not be allowed to dry out.)

9. Treat with 5% Normal Swine Serum for 5 min.

(Normal tissue contains other connective tissues such as collagen. These have an affinity for the antibody, non-specific binding. As a result these sites have to be blocked. To do this use whole serum from the appropriate animal i.e. use the serum

from the species that the secondary antibody has been raised in. This blocks out interference which can occur by the secondary antibody binding to sites in the tissue and swamping the antigen/antibody effect. After this treatment the secondary antibody should only bind to the primary antibody.)

10. Tap off. DO NOT WASH.

Tip off excess serum

11. Incubate in Primary Antisera for 1 hour at 37°C.

Allow 100 µl per small section. With larger sections may need to increase volume containing amount of antibody required accordingly. Use a 1.0 ml plastic pasteur pipette to aliquot antibody (a couple of drops \equiv 100 µl). Negative controls just get 100µl TBS.

N.B. Put the sections into the slide tray/box before you add the primary antibody. Put on the lid and leave in a warm place (or in the incubator at 37°C depending on assay). Must ensure that the sections do not dry out. If using DAKO tray make sure the reservoir has TBS in it. If using a box (Shandon) put a tissue soaked in TBS along the bottom of the box at the top of the glass slides (white border at the bottom so can read what is written on the slides).

12. Gently rinse in TBS from wash bottle.

13. Incubate in secondary antibody for 40 min at 37°C.

For polyclonals the secondary antibody is Swine Anti-Rabbit Biotinylated (Red). This has to be diluted 1/500 in TBS. Make up sufficient quantity so that there is 100 µl per section. Use a 1.0ml plastic pasteur pipette to aliquot diluted antibody (a couple of drops \equiv 100µl).

14. Prepare Avidin Biotin Complex (ABC).

ABC must be made up at least 30 min before use as it has to complex. Make up in the ratio 10:10:1000 A:B:TBS and store at 4°C until required. 1000 µl is sufficient for 5 sections.

15. Gently rinse twice in TBS from wash bottle.

16. Add ABC and leave at room temperature for 40 min.

Aliquot 200µl ABC per section with plastic pasteur pipette (about 4 drops).

Make up DAB during this incubation time. (DAB is very nasty so take care and make up and use solution in a fumehood.)

17. Gently rinse twice in TBS from a wash bottle.

18. Aliquot 200-300 µl of DAB onto each section and leave for 10 min.

19. Remove DAB with a disposable pasteur pipette into waste bottle. Rinse sections with 200-300 µl distilled water and remove this with pasteur pipette into waste bottle.

20. Place sections in a rack which is standing in a pot of tap water, then wash in running tap water for 5 min.

21. Stain nuclei in Gills haematoxylin for 1.0 min.

Can re-use Gills solution many times. Only need to change once staining begins to get faint. However if there is a shiny film on the surface remove this before you immerse the slide rack by using a folded tissue to skim the surface. If the Gills has been used a lot or left over the weekend filter it (filter paper and funnel is sufficient).

22. Blue in tap water. (Do this by placing rack in a pot of running tap water).

In Bath, because the local water supply is alkaline, all you have to do is wash in running tap water for about 10 mins. This causes the haematoxylin stain to turn blue. If in an area where the water is more acidic, need to use a solution of bicarbonate.

N.B. Empty first lot of water out immediately as otherwise the sections can effectively sit in haematoxylin longer if the concentration is greater at the bottom.

23. Dehydrate sections.

Dry off rack with a tissue. Dunk in industrial alcohol twice (a few seconds in each pot). Then put rack into another pot of citroclear. (Don't have to leave the sections in here too long. Just ensure that the citroclear on the sections runs clear before they are mounted.

24. Mount sections in XAM.

Place cover slips of suitable sizes onto tissue. Put a blob of XAM on the cover slip, using a 3.0 ml plastic pasteur pipette, one blob/section. Put slide, still 'runny' with citroclear (ie take slide out of rack which is still in the citroclear, stand slide up on end on pile of green paper towels just to drain off excess citroclear) on top of the coverslip. Turn slide over and allow weight of cover slip to spread out the XAM and displace the air bubbles. Any persistent air bubbles can be forced out from under the coverslip with a slight amount of pressure. Once all the coverslips are on can dry off with a cloth or tissue.

N.B. Make sure that you put the cover slips on the correct side of the slide.

IMMUNO PEROXIDASE SOLUTIONS

See Appendix 6.

PROTEIN DOT BLOTTING

1. Wearing gloves to avoid protein contamination, cut out a piece of nitrocellulose membrane and activate with methanol. (Membrane is white, once activated in methanol it goes opaque. Proteins will not bind if not activated.)
2. Wash in distilled water and place on a piece of clean filter paper to dry off excess water.
3. Dot membrane with the required concentration of the recombinant protein.
4. Cut off one corner of the membrane then make a small dot with a pencil on the membrane to mark where the protein is being dotted. (Need to dot the protein on in minute amounts. Once the dot is dry it is impossible to see where the protein is.) Make a note of where each protein has been dotted.
5. Once dots are dry place membrane into blocking buffer and agitate on a shaker for one hour.
6. Pour off blocking buffer and wash twice with NaCl/Tris, shaking for 10 min. Wash for a third time in NaCl/Tris + Tween for 10 min.
7. Cover membrane with approximately 10 ml of diluted antibody in NaCl/Tris + Tween. Shake at room temperature for two hours.
8. Pour off diluted antibody and wash membrane three times in NaCl/Tris + Tween, shaking for 10 min.
9. Cover membrane with approximately 10 ml of diluted alkaline phosphatase secondary in NaCl/Tris + Tween. Shake at room temperature for one hour.
10. Pour off diluted secondary antibody and wash membrane three times in NaCl/Tris + Tween, shaking for 10 min.
11. Add detection solution to the membrane and allow brown stain to develop (rapidly). Stop the reaction by quenching with cold PBS. Wash membrane twice in PBS
12. Leave to dry on clean filter paper.

PROTEIN DOT BLOTTING SOLUTIONS

BLOTTING BUFFER (NaCl/Tris)

170mM NaCl (9.93 g/l)

10mM Tris (TRIZMA base) (1.211 g/l)

Make up to 1000 ml with distilled water. pH to 7.4 with concentrated HCl.

BLOCKING BUFFER

5% low fat milk powder (50 g/l) in NaCl/Tris (blotting buffer)

NACL/TRIS + TWEEN

0.05% Tween-20 (0.5 ML/1000 ml of NaCl/Tris)

HIGH SALT WASH

100 ml NaCl/Tris + Tween + 1.93 g NaCl

DETECTION SOLUTION

33 µl Nitro blue tetrazolium (NBT)

33 µl 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)

Make up to 10 ml with

0.1M Tris	}	
0.1M NaCl	}	pH 9.5
50mM MgCl ₂	}	

N.B. 5-Bromo-4-chloro-3-indolyl phosphate does not dissolve well so mix until in suspension.

NBT = 100 mg/ml in 70% dimethylformamide

BCIP = 50 mg/ml in 100% dimethylformamide

SERUM HAPTOGLOBIN

1. Melt a 7.5 (15.0) ml aliquot of agarose, which is in a glass universal, in a beaker of boiling water on a hot plate. Transfer to a 56°C water bath when melted.
2. Weigh 0.15 (0.30) g of PEG 6000 into a plastic universal then add 7.5 (15.0) ml working buffer and place in the 56°C water bath.
3. Set up the levelling table and place a glass plate on it to insulate and support the plastic film (Gelbond).
4. Cut a piece of Gelbond to the size required, 10 cm × 10 cm (10 cm × 20 cm), and attach it to the glass plate (hydrophobic side down) using a thin film of water, ensuring all the corners are down.

N.B. The longer lengths of Gelbond tend to spring up away from the glass plate, overcoming the effect of the film of water. This also happens with the shorter lengths, towards the end of the roll where the plastic film, close to the middle, has been rolled very tightly. To prevent this happening, cut the required length of Gelbond, at least 48 hours before required, and roll it tightly in the opposite direction either around a suitable cardboard inner from a roll of paper or slip it inside a 100 ml measuring cylinder.

5. Quickly, but accurately, pipette 50 (100) µl of the anti-human haptoglobin into the warmed buffer. Invert to mix, and pour the contents into the glass universal containing the agarose. Mix by rolling gently in the hands (so no air bubbles form).

N.B. Keep buffer and agarose at 56°C as antibody will be destroyed if temperature goes much above this. However, need to have agarose as hot as possible so it will not set too soon when you pour it (that is before it has covered the entire surface of the plastic film).

6. Pour contents of glass universal into the middle of the Gelbond and touch the universal to the corners, if necessary, to produce an even layer.
7. Whilst the gel is setting, fill the buffer containers of electrophoresis tank to the maximum level, set up the cooling platen in the tank and prepare two wicks, using the glass plate as a template.

N.B. The wicks can either be made of Whatman filter paper, four pieces/wick, or as a cheaper alternative hospital lint can be used. Cut a single length of lint/gel, long enough so that it can be folded in half and the straight, folded, edge is used to overlap the gel.

8. Into plastic LP3 tubes aliquot sufficient working buffer for dilution of standards (90 μ l), samples (990 μ l) and control serum (990 μ l) then add 10 μ l of each of the sera to be assayed to the relevant tubes.
9. When the gel has set punch a row of wells, using a 5 μ l well punch, one well every 0.5 cm, positioning the row of wells 2.0 cm from the edge of the gel (using a piece of graph paper within a polythene cover as a template).

Instead of a well punch a glass tip from an Oxford Pipette can be used, connected via tubes, of varying diameters, to a Buchner flask which is itself connected to a venturi pump.

10. Place the glass plate with the gel onto the cooling platen, with the row of wells nearest the cathode, and carefully place the wicks on the gel so that they overlap by about 1.0 cm. (Make sure that the wicks are soaked in buffer and that the other ends are at least 5.0 cm below the level of the buffer.) Turn on the water to the cooling platen and power to 250 volts to equilibrate for 5 min.
11. Aliquot 5 μ l of standards, test samples and control serum into the wells, leaving the outer and any badly punched wells empty. (The larger gels need to have two sets of

standards, one each side, whereas one set of standards in the middle is sufficient for the smaller gels.)

12. Electrophorese for 4 hours at 250 volts or for 1 hour at 250 volts followed by 18 hours (overnight) at 110 volts. (The rockets are more distinct and have a better shape when the electrophoresis is conducted slowly.)

N.B. During high voltage electrophoresis the lid may need to be wiped clear of condensation every 30 min.

STAINING SERUM HAPTOGLOBINS

Switch off at the end of the electrophoresis time. Remove the glass plate from the cooling platen and place it agarose up on a piece of dry Whatman filter paper. Fill the wells with distilled water and place a piece of wet filter paper on top of the gel taking care to exclude any air bubbles (carefully use a glass pipette as a 'rolling pin'). Add 4 more layers of dry filter paper followed by some paper (green interlocked) hand towels and then place a large glass plate on the top. Place a heavy weight on top of the glass plate and leave to press for 15-20 min.

Remove layers of paper hand towel (the ones which are still dry can be re-used) and the pieces of filter paper, taking care not to peel the thin film of gel off with the paper. Dry the gel with a hair dryer until it becomes transparent and glossy.

In a fume hood, place gel in the stain until the rockets are clearly seen. Wash in de-staining solution (two or three times) until the de-stain solution is clear.

Blot with filter paper and then dry with a hair dryer.

Measure the rocket height from the top of the well to the tip of the rocket. Plot a graph of rocket height against \ln haptoglobin concentration of the standards using linear graph paper.

SERUM HAPTOGLOBIN SOLUTIONS

1. BARBITAL BUFFER 0.02M. pH 8.6

20.61 g Sodium Barbitone $[(C_2H_5)_2CCONHC(ONa):NCO]$ FW:- 206.18

4.00 g Barbitone (5,5-Diethylbarbituric acid) $[(C_2H_5)_2CCONHCONCCO]$ FW:- 184.20

Place a litre of distilled water in a glass beaker with a flea and set stirring on a heater/stirrer in a fume hood. Wearing a mask, weigh out the reagents in the fume hood, front well down but the fan off, and then add the fine powder to the distilled water. (If the reagents are not added while the water is stirring they form large clumps which take a long time to dissolve.) Switch on the fume hood and then heat the solution gently. Once the reagents have dissolved pour the contents of the beaker into a 5 litre volumetric flask and make up to 5 litres with distilled water. (There is no need to pH the solution.) Store at 27°C.

Barbitone BDH:- 27282

Sodium Barbitone BDH:- 27283

2. AGAR GEL

1.0 g

Dissolve the agarose in 50 ml of the Barbitol buffer 0.02M, pH 8.6, by boiling in a small glass beaker on a hot plate. Pour 7.5 (15.0) ml aliquots into glass universals and once cooled store at 4°C.

Agarose 15 'Electran' BDH:- 443023R

3. STAINS

Coomassie Blue Stain

0.4g Brilliant Blue R (Coomassie Brilliant Blue R-250) $[C_{45}H_{44}N_3O_7S_2Na]$ FW:- 826.0

80 ml Methanol

20 ml Glacial Acetic Acid

120 ml distilled water

Add the Coomassie Blue R to the distilled water and dissolve then add the solvents to this solution and mix.

Brilliant Blue R

Sigma:- B-0149

Methanol (HPLC Grade)

BDH:- UN1230

Glacial Acetic Acid

Fisons:-A/0360/PB17

De-Staining Solution

As above but without the Coomassie blue.

CHARACTERISTICS OF ULCERS OF THE SMALL BOWEL INDUCED BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN THE RAT: IMPLICATIONS FOR CLINICAL PRACTICE

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SUMMARY

Small bowel ulcers were created in the rat after the oral administration of non-steroidal anti-inflammatory drugs (NSAIDs). Of the six NSAIDs tested, indomethacin and diclofenac alone were associated with such damage which did not occur in a simple dose-related fashion. Bacteria were observed by electron microscopy in an active state of division in the base of the ulcers. When grown aerobically these were shown to be strains of *Escherichia coli* and *Proteus mirabilis*. Anatomically, NSAID-induced ulcers were found throughout the length of the bowel although more abundant in the proximal half. *In vivo* and *in vitro* sensitivity to antibiotics suggested that in addition to the bacteria identified, anaerobic β -lactamase-producing organisms also have an important role in ulcer production in this model. This rat model of NSAID-induced gut toxicity is discussed in relation to the human situation, particularly for patients who take NSAIDs and who have an iron-deficiency anaemia and blood in their faeces, but no lesions in either the upper or lower bowel.

KEY WORDS: Small bowel, Ulcers, Bacteria, Antibiotics.

THE effects of ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) in man and consequent damage to the upper gastrointestinal (GI) tract is well accepted and described [1]. Further, the toxicological mechanisms which lead to the breakdown of the gastric protective mechanisms is partially understood [2]. There is strong evidence to suggest that damage from NSAIDs is not apparently limited to the upper GI tract, but extends to the small bowel. Animal studies [3] predicted the finding of Bjarnason *et al.* [4, 5] who have described human experiments with NSAIDs, particularly indomethacin, which caused inflammation of the small bowel (SB) with leakage of cells and protein into the lumen of the order of that found in Crohn's disease. Visual evidence of this damage has been described by Morris *et al.* by enteroscopy [6, 7], and post-mortem studies have shown increased incidence of SB lesions after NSAID ingestion [8].

The rat model has been used to measure the damage caused by individual NSAIDs in the small bowel, and to explain the mechanisms involved. Inhibition of prostaglandin protection [9, 10], bacterial infection [11] and biliary excretion of NSAIDs [12, 13] have been implicated, but the relevance of these findings to the situation in the human small bowel has not been demonstrated. We have attempted to clarify the characteristics of SB NSAID-induced lesions in the rat in respect to their association with particular drugs, their position in the SB, their anatomical association with bacteria, the bacteria involved, and the sensitivity of the associated bacteria and SB ulceration to treat-

ment with antibiotics, in an attempt to see their relevance to the clinical situation, particularly that experienced by patients on long-term NSAIDs who become anaemic and who are suspected to harbour SB lesions.

MATERIALS AND METHODS

Animals and drugs

Male Wistar rats (Bath University strain) of 250–270 g body weight were used throughout. They were given free access to food and water at all times. Six NSAIDs were studied: indomethacin, diclofenac, naproxen, piroxicam, fenbufen and nabumetone. These were purchased from the Sigma Chemical Co. (Poole, Dorset), except for nabumetone which was a gift from SmithKline Beecham. The minimum dose (ID₂₅) of each NSAID used was the amount required to inhibit carageenan-induced oedema in the rat by 25% and the maximum was five times this dose. For oral dosing, the drugs were vortex mixed in Tween 80/water 1:9 and sonicated for 30 min. These drugs were administered using an oral-dosing needle in a volume of not more than 1 ml.

Antibiotic prophylaxis

Groups of three or more animals (per treatment) were used to demonstrate the prophylactic effect of antibiotics on the creation of SB ulceration which was induced by indomethacin given orally at a dose of 6 mg/kg on a single occasion. Three antibiotics were studied *in vivo*; metronidazole, amoxycillin and cloxacillin at doses of 50–100 mg/kg given either individually or in combination by the oral route once a day for 3 days before an acute ulcerogenic dose of indomethacin (6 mg/kg). The SB was examined 2 days later and damage measured as described below.

Submitted 30 December 1994; revised version accepted 27 April 1995.

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Acute SB ulceration

After acute NSAID administration, rats were killed after 48 h. The gut was opened along the anti-mesenteric side and divided into 15-cm lengths which were displayed in sequence. SB lesions were identified and copied onto an overlaid acetate sheet.

Scanning electron microscopy (SEM)

Individual ulcers were identified and excised with a margin of normal mucosa, under aseptic conditions. The tissue was fixed in a 2% glutaraldehyde/0.05 M phosphate buffer, pH 7.4, for 24 h and post-fixed with osmium tetroxide in 0.1 M phosphate/0.15 M sucrose buffer followed by the OTOTO method of staining [14, 15].

Bacteriological culture

Ulcer bases were sampled aseptically and inoculates made into Tryptone broth or Thioglycolate medium USP, and incubated at 37°C for 48 h. Gram-negative bacteria were identified using an api 20E kit (bio Mérieux). Isolation of strictly anaerobic organisms was not attempted. Cultures of the bacteria obtained from ulcer bases were partially characterized and their growth tested against a range of antibiotics active against aerobic and anaerobic organisms.

RESULTS

Toxicity of individual NSAIDs

Of the six NSAIDs tested, only indomethacin and diclofenac caused SB lesions. The other four NSAIDs failed to produce SB lesions even when the highest

dose was administered chronically over a period of 28 days.

Characteristics and distribution of SB damage

Both diclofenac and indomethacin caused similar types of damage. An acute dose of indomethacin at a dose of 7.5 mg/kg caused acute haemorrhage, oedema and perforation of the SB in the area of the jejunum within 24 h of administration. At lower doses, indomethacin caused discrete ulcers throughout the small bowel and/or a thin longitudinal lesion of up to 10 cm in length. Diclofenac caused lesions similar to those seen with indomethacin but only after four daily doses of 10.35 mg/kg. These lesions were true ulcers (Fig. 1). Their distribution in the SB is shown in Fig. 2.

Characteristics of SB ulcers by SEM

Characteristically ulcer bases showed the presence of many bacteria in active division, while adjacent mucosa showed few if any organisms. The bacteria (Fig. 3) were shown to be a mixture of *Escherichia coli* and *Proteus mirabilis*, although strict anaerobes were not identified by the culture methods used.

Prophylactic effect of antibiotics on NSAID-induced SB ulceration

Metronidazole (100 mg/kg) and amoxycillin (50 mg/kg) given orally for three days before the administration of indomethacin (6 mg/kg) on day four, completely abolished its ulcerogenic effect. When the interval between the last antibiotic dose and indomethacin exceeded 7 days, the prophylactic protection was lost. Control animals dosed with saline in

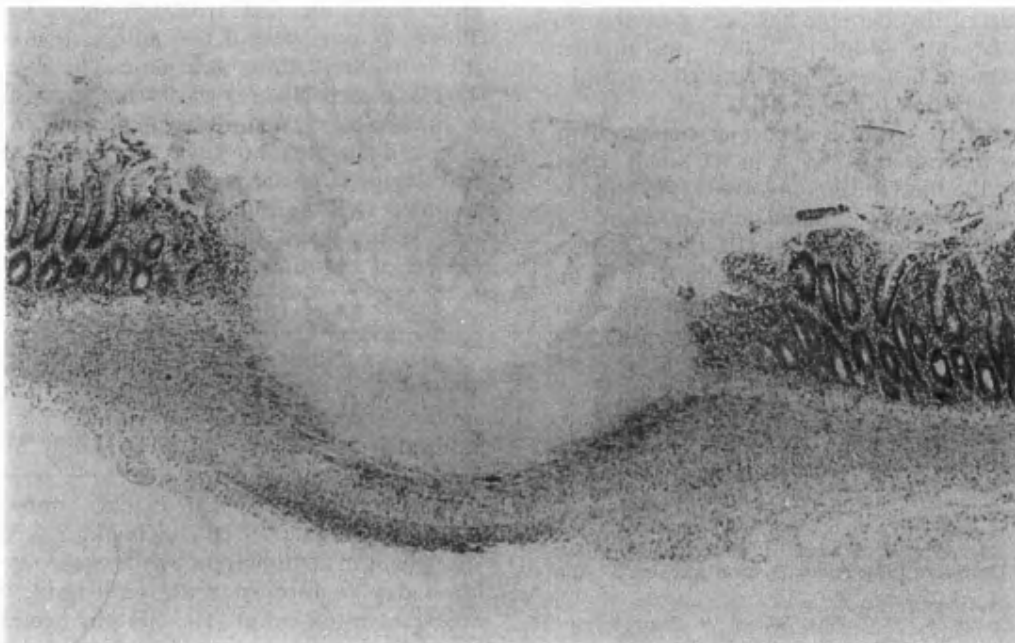


FIG. 1.—The histological appearance of a rat small bowel ulcer 48 h after induction by an acute oral dose of indomethacin (6 mg/kg). The section shows the lesion penetrates the full thickness of the mucosa, and is a true ulcer rather than an erosion.

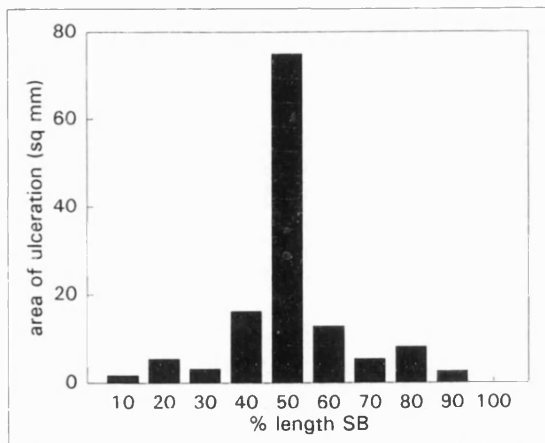


FIG. 2.—The distribution of ulceration (in mm²) along the small bowel of the rat given diclofenac (10.35 mg/kg orally, daily for 4 days). The figure shows the mean result from six animals killed 48 h after the last dose. Bowel length is expressed as a percentage distance from the stomach to ileocaecal junction to adjust for variation in individual bowel length.

place of the antibiotics showed the expected pattern of ulceration throughout the experiment. Animals treated prophylactically with amoxycillin, metronidazole and cloxacillin given individually all produced a reduction in ulcer frequency after an indomethacin challenge. Metronidazole and cloxacillin protected the terminal SB, amoxycillin tended to protect the proximal SB (Fig. 4).

Differential effects of antibiotics on SB ulceration and on associated bacteria grown in vitro

When a number of antibiotics were tested against the *in vitro* growth of organisms recovered from SB ulcer bases collected as described, differences in inhibitory effects were demonstrated (Table I). Amoxycillin was an effective inhibitor. Metronidazole (100 mg/kg) and cloxacillin (100 mg/kg) were ineffective in inhibiting growth of mixed cultures of *E. coli* and *P. mirabilis* although they were active in preventing ulcers *in vivo*, suggesting that essential anaerobic bacteria had not been isolated by the methods described, and were probably the organisms necessary for ulcer formation.

DISCUSSION

If the rat model of NSAID-induced SB damage is to be useful in understanding the clinical phenomenon of anaemia associated with the ingestion of NSAIDs, suppositions must be made concerning the pathogenesis and pathology of the SB damage in the two situations. There are obvious differences.

The time course of the damage appears to differ. In humans, Bjarnasson *et al.* have suggested a long exposure to NSAIDs is necessary for an enteropathy and blood loss to occur [4, 5]. The SB in the rat model ulcerates within hours or days of exposure to specific NSAIDs, with acute perforation of the jejunum at high doses. Enteroscopic determination of SB ulceration in man has been retrospective with no clear association of the time course of action of these drugs and with no association with particular NSAIDs [6, 7]. Lesions in

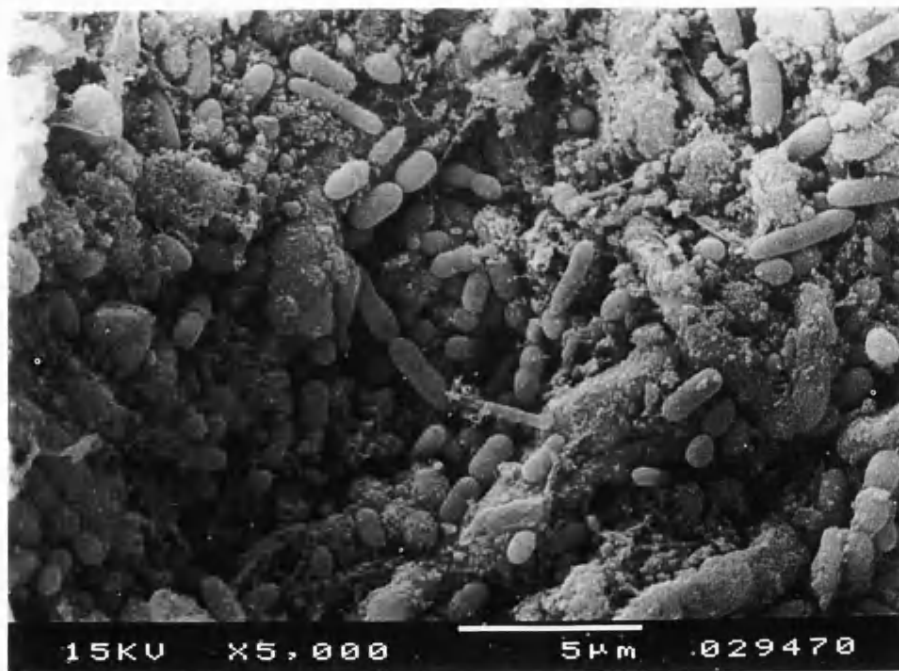


FIG. 3.—Scanning electron micrograph of a representative small bowel ulcer formed after the oral administration of diclofenac (10.35 mg/kg orally, daily for 4 days). The ulcer base was covered with a dense collection of actively dividing bacteria; most were short rods, many with newly formed septa, some with features suggesting L forms. Adjacent areas of non-ulcerated mucosa showed only scant growth of bacteria. When grown aerobically these bacteria were shown to be *Escherichia coli* and *Proteus mirabilis* spp.

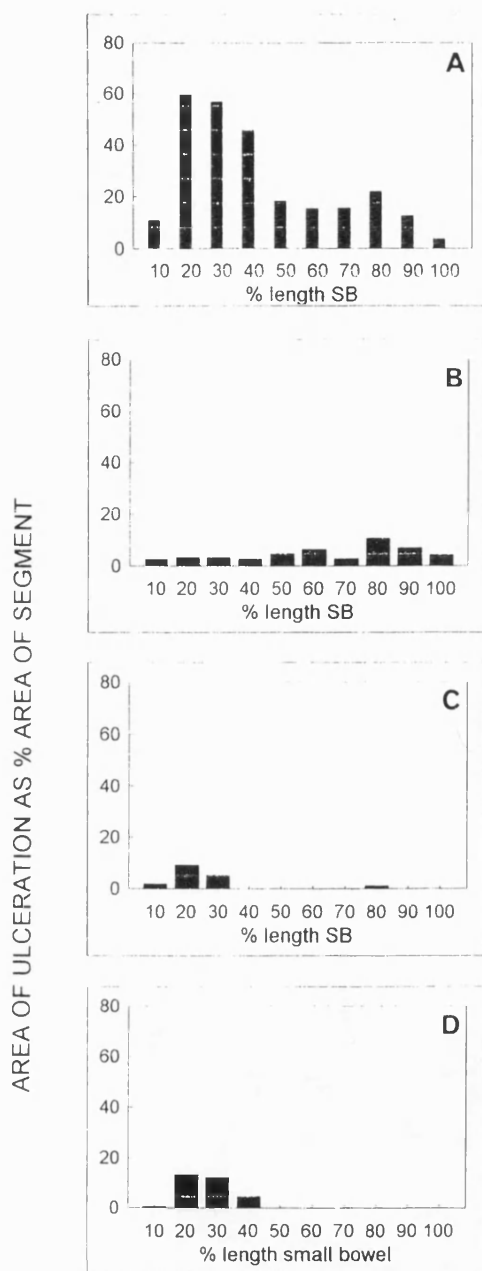


FIG. 4.—The comparative action of a variety of antibiotics in inhibiting indomethacin-induced small bowel ulcers in the rat. Bars represent the mean of six animals per experiment. (A) Control: distribution and number of small bowel ulcers after a single oral dose of indomethacin (6 mg/kg). (B) Prophylactic dose of amoxycillin (50 mg/kg) given once a day for 2 days before the indomethacin challenge. (C) Cloxacillin given as in (B) at a dose of 100 mg/kg. (D) Metronidazole given as in (B) at a dose of 100 mg/kg.

the rat SB were always true ulcers whereas enteroscopic views of the human SB often reported haemorrhagic lesions, probably erosions.

The creation of ulcers in the rat SB depended absolutely on the presence of bacteria, probably anaerobes for their production. These bacteria were likely to be of faecal origin and their source was probably

ingested faeces, given the rat's habit of coprophagia, explaining the presence of viable coliform organisms in ulcer bases throughout the whole length of the SB. This would not be expected in the human SB and thus ulcers, if present, may be caused by another mechanism. Recent experiments carried out by us have not detected coliform organisms in the SB of patients who take NSAIDs and who have anaemia, but have shown the presence of other organisms [16].

When patients with an enteropathy and blood loss ascribed to a NSAID were given metronidazole, the result was to reduce blood loss suggesting either an antimicrobial effect or a direct action of the drug on the ulcerogenic process [17]. Our results suggest only prophylactic treatment protects against NSAID ulcers. Antibiotics given following ulcer formation had no effect [18]. Our data from *in vitro* and *in vivo* effects of antibiotics (Table I; Fig. 4) show that metronidazole and cloxacillin, which have no effect on the inhibition of gut bacteria grown aerobically, nevertheless prophylactically protect against NSAID-induced ulcers *in vivo*. The organisms involved in ulceration are therefore likely to be strict anaerobes and there is no direct effect of the antibiotic on the ulcerogenic process *per se*.

It has been suggested that NSAIDs that are excreted via the bile are particularly prone to cause SB damage [12, 13, 19, 20], perhaps because of the extra-enteric exposure this produces. In the rat however, other NSAIDs at high doses did not result in ulceration, suggesting not the primary NSAID but a metabolite may be the initiator of damage. To extrapolate these findings to man requires the hepatic metabolites of NSAIDs to be quantitatively and qualitatively similar in both species.

The rat SB is sensitive to the effects of NSAIDs, but peculiarly only indomethacin and diclofenac, and at relatively high therapeutic doses. Their effects appear to be completely dependent on a bacterial adjuvant before ulceration is manifest. It is unlikely that the human situation can be directly compared with this model in respect to either specific NSAIDs, their dose or therapeutic protective measures. The extrapolation

TABLE I

The comparative inhibition caused by a variety of antibiotics when applied to a mixed culture of organisms taken from an ulcer base represented by Fig. 4. The organisms grown aerobically were *E. coli* and *P. mirabilis*. The comparative figures of inhibition were produced using oxoid antimicrobial susceptibility discs on oxoid antimicrobial test media cultured at 37°C

Antibiotic	Oxoid code	Zone (mm) of inhibition
Ciprofloxacin	CIP 5	10.8
Amoxycillin	AML 25	7.3
Cefuroxime	CXM 30	5.8
Neomycin	N 30	5.5
Nalidixic acid	Na 30	4.8
Chloramphenicol	C10	4.3
Gentamicin	CN 10	3.5
Oxytetracycline	OT 30	3.3
Metronidazole	MTZ 50	0
Cloxacillin	OB 5	0

of animal data to the clinical situation is therefore likely to be of limited value.

ACKNOWLEDGEMENTS

We are grateful to SmithKline Beecham who supported this work, and Ursula Potter, Electron Optics Unit, University of Bath for electron microscopy.

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